DETERMINATION AND TOXICOLOGICAL EVALUATION OF MICROCYSTINS IN TROPICAL RESERVOIRS

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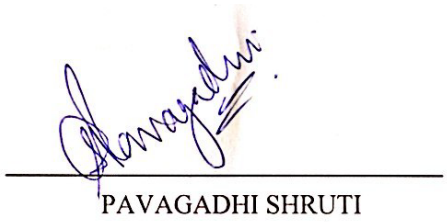
DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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SUMMARY

Micocystin-LR (MCLR) and Microcystin-RR (MCRR) are cyanotoxins that are produced by blue-green algae. These cyanotoxins are found in eutrophic aquatic systems. They are known as hepatoxins as they are known to damage liver. MCLR and MCRR are the most commonly found and studied microcystins (MCs). Trace levels of these MCs sustain extracellularly for sufficiently long periods in contaminated water bodies even when the environmental conditions are not favorable for the growth of toxic cyanobacteria. As a consequence, there are concerns about the potential health effects of extracellular MCs to organisms living in aquatic ecosystems and thus to humans as well.

Although MCLR and MCRR may exist in sub-lethal concentrations in water phase, sub-chronic and chronic balneation (i.e. when organisms are bathed in the water containing the toxins) exposure can often lead to deleterious health effects to the complete ecosystem. This health concern calls for a systematic assessment of potential hazards associated with the release of MCs in the aquatic fresh water environments, following lyses of harmful algal blooms (HABs). This risk assessment is of prime importance in the local as well as regional context in Southeast Asia since anthropogenic activities due to high population growth and rapid urbanization have increased tremendously in the past few decades. Anthropogenic release of nutrients (nitrogen and phosphorous) into the aquatic systems could lead to increased eutrophication and thus the occurrence of HABs. Frequent HABs could in turn lead to sustained exposure of extracellular MCs to aquatic organisms, particularly fish which are not only ideal for water quality monitoring, but also act as an indirect link between the aquatic and human habitats.

However, at present, there is a lack of in-depth knowledge available for toxicological implications of extracellular MCs to aquatic organisms. Hence, the present doctoral study was designed to fill these knowledge gaps. The gaps were fulfilled through a series of systematic investigations made under this doctoral study. Entire study was divided in two components: Analytical and Toxicological.

Under analytical component, an environmental friendly method for extraction and analyses of MCLR and MCRR from natural waters was developed. Under the toxicological component, attempt was made to understand the toxicological implications of extracellular MCLR and MCRR on aquatic organisms. Zebrafish was selected as a model organism for this doctoral study. Detailed investigations resulted in interesting outcomes suggesting the role of MCLR and MCRR beyond being mere 'hepatoxins' as perceived by the scientific community. Experiments from this study also resulted in identification of toxicological markers/ biomarkers which could be attributed to balneation exposure of zebrafish to MCLR/MCRR. Controlled experimental exposure using relevant concentrations of MCLR and MCRR was carried out for the identification of the same.

LIST OF PUBLICATIONS

This doctoral study resulted in the following research publications

Peer-reviewed articles in international journals

1. **S. Pavagadhi**, Z. Gong, M.P. Hande, D.D. Dionysiou, A.A. de la Cruz and R. Balasubramanian. Biochemical response of diverse organs in adult *Danio rerio* (zebrafish) exposed to sub-lethal concentrations of microcystin-LR and microcystin-RR: A balneation study. Aquatic Toxicology, 109 (2012), 1-10 (**IF: 3.761**).
2. **S. Pavagadhi**, C. Basheer, R. Balasubramanian. Application of Ionic-liquid supported Cloud Point Extraction for the Determination of Microcystin-LR in Natural Waters. Analytica Chimica Acta , 686 (2011), 87-92 (**IF : 4.55**)
3. M. Sathishkumar, **S. Pavagadhi**, K.Vijayaraghavan, R. Balasubramanian, S.L.Ong. Concomitant uptake of microcystin-LR and -RR by peat under various environmental conditions. Chemical Engineering Journal, 172 (2011), 754-762 (**IF: 3.46**)
4. M. Sathishkumar, **S. Pavagadhi**, R. Balasubramanian. Experimental Studies on Removal of MCLR by Peat. Journal of Hazardous materials, 184 (2010), 417-424 (**IF: 4.17**)
5. M. Sathishkumar, **S. Pavagadhi**, A. Mahadevan, R. Balasubramanian, D. Burger. Removal of potent cyanobacterial hepatotoxin by peat. Journal of Environmental Science and Health Part A, 45 (2010), 1877–1884 (**IF:1.36**)
6. **S.Pavagadhi**, R. Balasubramanian. Global gene expression profiling in adult zebrafish exposed to MCLR/MCRR reveals endrocrine disrupting effects of microcystins *(in preparation).*
7. **S.Pavagadhi,** R. Balasubramanian. Disruption of lipid and carbohydrate metabolism in adult zebrafish following a balneation exposure to MCLR/MCRR *(in preparation).*

Conference Proceedings

1. **S. Pavagadhi**, G. Zhiyuan, U. Roessner, S. Natera and R. Balasubramanian. Evaluation of cellular and tissue level biomarkers in *Danio rerio* on exposure to MCLR and MCRR. 2nd Australasian Symposium on Metabolomics, Melbourne, Australia (26-28th September, 2010)
2. **S. Pavagadhi**, G. Zhiyuan, R. Balasubramanian. Uptake and Effects of Microcystin-LR and Microcystin-RR on Antioxidant and Detoxification Enzymes

of *Danio rerio* (zebra fish). 8th International Conference on Toxic Cyanobacteria (ICTC 8), Istanbul, Turkey, (29th August-4th September 2010)

3. M. Sathishkumar, **S. Pavagadhi**, and R. Balasubramanian. Single and dual-component removal of microcystin–LR and –RR from aqueous phase using peat.. 8th International Conference on Toxic Cyanobacteria (ICTC 8), Istanbul, Turkey, (29th August-4th September 2010).
4. **S.Pavagadhi,** R.Balasubramanian (2009) Novel Approach for Detection of Cyanobacterial Toxins from Natural Waters. Presented at Global Conference on microbial contaminants in drinking water, Singapore (6-8th October 2009).

Book Chapter

Practices that prevent the formation of cyanobacterial blooms in water resources and cyanotoxins during water treatment. M. G. Antoniou, M. A. Pelaez, W. Song, K. O'Shea, L. Ho, G. Newcombe, M. R. Teixeira, A. A. de La Cruz, T. M. Theodoros, T. Kaloudis, A.Hiskia, R. Balasubramanian, **S. Pavagadhi**, C. Han, V. Sharma, M. Dixon, X. He, and D. D. Dionysiou. In Comprehensive Water Quality and Purification. Elsevier Press, UK, 2012 *(in press)*.

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LIST OF ABBREVIATIONS

ADDA	(2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BMiM PF_6	1-butyl-3-methylimidazolium hexafluorophosphate
BSA	Bovine Serum Albumin
C 18	Octadecyl Silica
cDNA	Complimentary deoxyribonucleic acid
CDNB	1-chloro-2, 4-dinitrobenzene
CE	Capillary Electrophoresis
CHABs	Coastal Harmful Algal Blooms
cRNA	Complimentary ribonucleic acid
CYP	Cytochrome P450
DAB	3,3- diaminobenzidine tetrahydrochloride
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
DTNB	5, 5-dithiobis (2-nitrobenzoic acid)
EDTA	Ethylenediaminetetraaceticacid
ELISA	Enzyme-linked immunosorbent assay
emM	Extinction Coefficient
ESI	Electrospray Ionization
ETC	Electron Transport Chain
FGF	Fibroblast growth factor
FHABs	Freshwater Harmful Algal Blooms
GI	Gastrointestinal tract
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Reduced glutathione
GSSG	Oxidised glutathione
GST	Glutathione-S-transferase
HABs	Harmful Algal Blooms
HLB	Hydrophilic–Lipophilicbalanced
HMIM PF_6	1-Hexyl-3-methylimidazolium hexafluorophosphate
HPLC	High-performance liquid chromatography
HUFA	Highly unsaturated fatty acids
IHC	Immunohistochemistry
IL/W	Ionic liquid/water distribution coefficients

IP	Intraperitoneal
LC-MS	Liquid chromatography/mass spectrometry
LD50	Lethal to half the test population
LOD	Limits of Detection
LOQ	Limit of Quantification
LPS	Lipopolysaccharides
MALDI-TOF-MS	Matrix Assisted Laser Desorption/ionization Time-of-Flight Mass Spectrometry
MAPK	Mitogen-activated Protein Kinases
MCLF	Microcystin- Leucine-Phenyl alanine
MCLR	Microcystin-lcine-arginine
MCRR	Microcystin-arginine-arginine
MCs	Microcystins
MIPs	Molecularly Imprinted Polymers
MRM	Multiple Reaction Monitoring
mRNA	Messenger Ribonucleic acid
NAPDH	Nicotinamide adenine dinucleotide phosphate
Nrf2	factor (erythroid-derived 2)-like 2
O/W	Octanol/water distribution coefficients
OD	Optical Density
OMIM PF_6	1-Octyl-3-methylimidazolium hexafluorophosphate
PBST	Phosphate buffered saline with tween
PCR	Polymerase Chain Reaction
PP1	Protein Phosphatase 1
PP-2A	Protein Phosphatase 2A
PPIA	Protein phosphatase inhibition assay
RA	Risk Assessment
RILs	Room temperature ionic liquids
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
RP-LC-MS	Reversed phase- Liquid chromatography – Mass spectrometry
RP-LC-q-TOF	Reversed phase-liquid chromatography-quadrupole time of flight
RSDs	Relative Standard Deviation
S/N	Signal to noise ratio
SEC	Size Exclusion Chromatography
SIM	Selected Ion Monitoring
SOD	Superoxide dismutase
SPE	Solid-Phase Extraction
SPME	Solid Phase micro-extraction
SRM	Selected Reaction Monitoring

TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor
UV	Ultraviolet
Vtg	Vitellogenin genes
WHO	World Health Organization
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

1.1 Background

Although cyanobacteria (blue-green algae) in water pose a health hazard to humans, they have been long neglected in scientific research. However, in recent years, there have been a number of harmful algal bloom (HABs) episodes involving toxic cyanobacteria in fresh waters stored in lakes which caused a variety of water-quality problems. Scums of these toxic cyanobacteria accumulating along the lakes and reservoirs also present a hazard to wild and domestic animals. There are a number of factors that affect the scum and bloom formation including sunlight intensity, availability of nutrients especially nitrogen and phosphorus, ambient temperature, etc. Under favorable conditions such as an excessive supply of nutrients, HABs are prone to occur. These HABs not only lead to strong odor formation and practical nuisance, but also limit the availability of dissolved oxygen and nutrients to the existing ecosystem in the contaminated water body. In addition, these scums resulting from the excessive growth of cyanobacteria may also lead to cyanotoxin production. The production of algal toxins is of serious health concern. Cyanotoxins in general are secondary metabolites, produced intracellularly within the cyanobacterial cells.

Among cyanotoxins, microcystins (MCs) which belong to the family of hepatotoxins have received special attention as they are the most commonly occurring in aquatic bodies among other cyanotoxins. MCs are monocyclic heptapeptides composed of seven

amino acids containing an unusual amino acid (2S, 3S, 8S, 9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) that is essential for the expression of biological activity. MCs are known for their ability to cause both acute and chronic health effects to humans through the consumption of contaminated drinking water and fish. MCs are known to bind covalently to protein phosphatases, thus disrupting the cellular control processes. These enzymes remove phosphate from a protein, a common step required in activation/deactivation for many biochemical pathways. This inhibition, with a subsequent build up of phosphorylated proteins, is believed to be a mechanism by which MCs destroy livers. There are numerous reports in the literature, describing a variety of health effects after exposure to extracellular MCs in drinking water, or from swimming in water in which toxic cyanobacteria are present. The most common sign of human poisoning with MCs is liver damage. Early manifestations of liver damage include an increase in serum of liver enzymes, a sign of liver cell death, and increased liver weight.

This health concern in conjunction with frequent occurrence of cyanobacterial blooms worldwide makes production of these toxins in natural waters a serious public health issue. At present, about 80 MCs have been identified across the world. However, MCLR (microcystin-leucine-arginine) and MCRR (microcystin-arginine-arginine) are the most commonly studied MCs. MCLR has been documented as the most lethal MC among all of the known MCs. A provisional safety guideline of 1.0 µg L^{-1} MCLR in drinking water was recommended by World Health Organisation (WHO). Most of the knowledge currently available in the literature about the toxicity of MCs is based on studies with

mice and rats that received intra-peritoneal (IP) injections of MCLR, i.e. injections directly into the abdominal cavity.

Both MCLR and MCRR are produced by a variety of cyanobacteria including *Microcystis, Anabaena, Oscillatoria, Nostoc, Anabaenopsis*, etc. These MCs occur as intracellular secondary metabolites. Upon bloom lyses, the toxins are released in the water systems and exist as extracellular MCs. MCs are extremely stable and resist common chemical breakdown such as hydrolysis or oxidation under conditions found in most natural water bodies. These toxins can break down slowly at high temperature (40 °C) at either very low (<1) or high (>9) pH. The half-life, the time it takes for one-half of the toxin to degrade, at pH 1 and 40ºC is 3 weeks; at typical ambient conditions, the half-life is 10 weeks. MCs break down slowly in full sunlight, especially when water-soluble pigments are present. As a consequence, they tend to persist for longer periods of time. With their persistence in the natural environment, there is a likelihood of chronic and sub chronic exposure of these extracellular MCs to the aquatic organisms present in the water body. As a result of this exposure, there is a possibility of the toxin accumulation in the aquatic food web. It has been shown that certain groups of organisms (e.g. protozoa, rotifers) are highly sensitive to these toxins which implies that processes such as microbial loop are disturbed with negative consequences for other organisms in the food web (e.g. fish, crustaceans). In that situation, there would be concerns over possible bioaccumulation and biomagnification at a higher level. Since commonly used water treatment technologies are not able to effectively remove these toxins to below acceptable levels, proper risk assessment and management initiatives

should be developed and employed to prevent the exposure of living forms to these lethal toxins.

However, with continual exposure, organisms may adapt to the stress conditions and avoid the consequences of exposure. Therefore, there is a need to evaluate the toxicological impacts of extracellular MCs in a holistic way. For risk assessment of any contaminated water body, it is of foremost importance to develop a sensitive analytical method to detect MCLR and MCRR from local water systems. In addition, to understand the potential deleterious health effects, preliminary biochemical tests as well as detailed toxicological studies are critically needed. All these scientific investigations should be conducted with local relevance in order to adopt realistic contingency plans.

Biochemical and toxicological endpoints could be evaluated using high-throughput approaches, which could lead to the identification of novel biomarkers that are specific to MCs exposure. Biomarkers could further aid in exposure and effect-assessment studies and also provide a scientific basis for monitoring MCs at a biological level.

1.2 Research Objectives

There have been many reports on method development for detection of intracellular and extracellular MCs in the aquatic medium. However, conventional and current methodologies require the use of organic solvents, some of which are toxic and environmentally unfriendly. In addition, these methods are often time consuming with separate extraction and concentration steps being carried out prior to detection. There is a need to develop robust, efficient and greener methodologies for extraction of MCs. Conventional methods for extraction and pre-concentration are often cumbersome, and

for routine monitoring purposes, simpler one step extraction/concentration step is often desirable. Hence, there is a need for developing a novel analytical method that can overcome the practical problems associated with the use of conventional extraction and detection techniques. The use of an appropriate analytical method is the most important consideration for risk assessment of any water body.

Apart from analytical method development, the toxicological impacts of extracellular MCLR and MCRR on any aquatic organism have not been fully understood yet. The toxicological impact of MCs has been demonstrated in a variety of organisms, but the exposure is largely limited to intracellular MCs. There are very limited reports in literature on exposure of aquatic organisms in a balneation episode, i.e., extracellular MCs. For complete and holistic risk assessment, both intracellular and extracellular MCs need to be fully evaluated for their toxic potencies. While the former type of MCs mainly contributes to acute toxicity, the latter one could lead to sub-chronic and chronic toxicities. Once the toxic potentials are evaluated under various environmentally relevant conditions, realistic contingency plans can be formulated. From the toxicological endpoints, one could identify potential biomarkers which are specific to extracellular MCs exposure. These biomarkers can be used for biomonitoring purposes for risk management.

To the best of our knowledge, there has been no study that reported specific biomarkers relevant to exposure to extracellular MCs present in aquatic organisms. MCs have been investigated for biochemical biomarkers in many organisms. However, research is still in its infancy for evaluating specific biomarkers pertaining to extracellular MCs exposure.

This study proposes a series of systematic investigations to fill the current knowledge gaps in the detection and toxicological evaluation of MCLR and MCRR present in tropical reservoirs. These investigations are designed to be mutually complementary to each other. The following specific aspects of research pertaining to MCLR and MCRR have been conducted in this study.

Specific Research Objectives:

1. Development and validation of a novel analytical method for rapid detection of MCLR and MCRR in tropical reservoirs
    - To identify and use a novel solvent for extraction of MCLR and MCRR;
    - To develop a green method for efficient and effective extraction/preconcentration of MCLR and MCRR;
    - To demonstrate the applications of the developed method for real water quality monitoring.
2. Evaluation of toxicological implications of extracellular MCLR and MCRR in zebrafish (*Danio rerio*) under balneation conditions
    - To understand biochemical changes at a cellular level:
        - Antioxidant enzymes : Non specific responses in various tissues
    - To understand metabolic changes occurring at an organ level:
        - Metabolite analysis (Metabolomics) : Specific responses in various tissues
    - To understand genomic changes occurring at a molecular level :
        - Transciptome analysis (Trancriptomics): Specific responses in various tissues.
    - To study the bio-distribution of MCs at a histological level:
        - Immunohistochemistry: Qualitative bio-distribution of MCLR and MCRR under balneation conditions.

3. Identification of possible biomarkers using various approaches

    - Creating biomarker networks using pathway analysis;
    - Identifying potential biomarker candidates for biomonitoring of extracellular MCLR and MCRR using zebrafish.

1.3 Organization of Thesis

The present doctoral study is organized into 10 chapters including 5 technical chapters. The general scope of each chapter is briefly discussed below:

Chapter 2: Literature Review

This chapter gives a brief overview of the current knowledge available in the literature in the research area pertaining to this doctoral thesis work. This chapter provides general background information for the research undertaken and also points key knowledge gaps that need to be filled.

Chapter 3: Materials and Methodology

This chapter enlists the key experimental methodologies and materials used throughout the course of this research study. Specific details on experimental framework are also provided.

Chapter 4: Development of an analytical tool for detection of MCLR and MCRR

In this chapter, a cloud point extraction method is described using an ionic liquid (1-butyl-3-methylimidazolium hexafluorophosphate (BMiM PF_6) for the determination of MCLR and MCRR in natural waters. The optimization of extraction parameters such as

sample pH, extraction temperature, extraction time, the amount of ionic liquid and the amount of extraction volume is discussed. In addition, the quantitative data obtained from determoination of MCLR and MCRR in local water samples are presented.

Chapter 5: Biochemical changes in zebrafish organs upon a balneation exposure

This chapter presents a study on dose-response of MCLR and MCRR on biochemical parameters in adult zebrafish under balneation conditions at various time points. The differential responses of superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) as biomarker antioxidant enzymes were assessed for oxygen mediated toxicity in liver, gills, intestine and brain tissues of zebrafish exposed to dissolved MCLR and MCRR (0.1 µg L^{-1} to 10.0 µg L^{-1}). To investigate the time related response of biomarkers, fish were sampled after 4, 7 and 15 days of exposure. Results obtained from this investigation are presented and discussed in the chapter.

Chapter 6: Immunohistochemistry for localized bio-distribution of MCLR and MCRR in zebrafish.

Chapter 6 describes a staining technique that was developed to study the qualitative bio-distribution of MCLR and MCRR in adult zebrafish tissues. The technique used for staining was immunohistochemistry. The principle underlying this technique is antibody binding to specific antigens in biological tissues. An antibody specific to ADDA moiety in MCs was used in this experiment to detect the presence of MCLR and MCRR in fish

tissues. Images, captured using a microscope fixed with a camera lens, are provided in the technical chapter.

Chapter 7: Metabolic changes in zebrafish organs upon a balneation exposure.

In this chapter, metabolite profiling (untargeted and targeted) describing the effects of MCLR and MCRR exposure on adult zebrafish under balneation conditions (10 µg L^{-1} for 30 days) has been described. Polar and non polar metabolites were detected and identified using high throughput analytical platforms. The profiling was done for various tissues of adult zebrafish. The aim behind these experiments was to identify specific biomarkers of interest upon exposure to extracellular MCLR and MCRR. Pathway mapping was done to understand the underlying mechanism of toxicity through metabolite markers.

Chapter 8: Transcriptome changes in zebrafish organs under a balneation exposure

Chapter 8 discusses the global gene expression analyses performed on adult zebrafish organs. RNA samples after exposure to MCLR and MCRR under balneation conditions (10 µg L^{-1} for 30 days). Along with metabolite profiling, these experiments form another higher level of biomarker sets. Based on the outcome of these experiments, specific biomarkers are listed at the molecular level. The objective behind gene expression studies was to gain a deep understanding of the changes occurring due to MCLR and MCRR toxicities at the molecular level. This work on gene expression would not only help in obtaining potential biomarkers, but also provide deep insights into the mechanisms of

toxicity and target biochemical pathways for MCLR and MCRR exposure. Pathway mapping was also done with these data sets to understand specific responses.

Chapter 9: Summary and Conclusions

Chapter 9 presents the outcome of the study undertaken in a logical sequence. The toxicological implications of exposure to extracellular MCs under balneation conditions are highlighted.

Chapter 10: Future recommendations

Recommendations for future work in this thesis area are provided such that the existing knowledge-base on extracellular MCs and their health impacts can be further expanded from the scientific research standpoint and practical strategies can be formulated for enhancing the quality of natural waters.

CHAPTER 2

LITERATURE REVIEW

2.1 Harmful Algal Blooms

The accumulation of cyanobacterial biomass as bright green, yellow–brown and red blooms in fresh, brackish or saline waters is one of the most obvious and problematic symptoms of anthropogenic nutrient enrichment, or eutrophication (Reynolds and Walsby, 1975).

Harmful algal blooms (HABs) have emerged as a worldwide concern due to the increased frequency of occurrence with severity, and the known acute and chronic toxicity to animals, plants and humans (Van Liere and Mur, 1980).

HABs are caused by massive and prolonged overgrowth of algae and other plant-like organisms such as dinoflagellates, diatoms and cyanobacteria (bacteria capable of photosynthesis). Growth and proliferation of these algal forms not only depend upon the supply of nutrients (nitrogen (N) and phosphorous (P)) but also on geographical and environmental factors. Some of the important factors that affect the cyanobacterial bloom formation are light intensity (500 - 650 nm), nitrogen to phosphorus ratio (10:1-16:1) and temperature distributions (20-35ºC) as a function of depth (Van Liere and Mur, 1980; Schreurs, 1992; Robarts and Zohary, 1987). The interplay of these factors could lead to excessive growth of cyanobacteria leading to a cyanobacterial bloom. Of major concern is over-enrichment of natural waters with N and P, a condition also known as eutrophication (Smith, 2008). There are a number of sources from which these nutrient

can be introduced into natural waters including geological weathering, non point exports and point sources exports from various human activities such as excessive use of fertilizers, pesticides and synthetic chemicals and untreated sewage effluents (Smith,2009). In view of the increased release of nutrients from these sources, incidence of HABs is widely reported in different parts of the world.

HABs appear to be increasing in the surface waters worldwide. Many countries are faced with a bewildering array of toxic or harmful species and impacts, as well as disturbing trends of increasing bloom incidence, larger areas affected, more fisheries resources impacted, and higher economic losses. HABs cause substantial, but unquantified amounts of human and animal morbidity and mortality from exposures in recreational, commercial, drinking-source and potable waters (Dionysiou, 2010).

The extensive occurrence of HABs can create considerable nuisance for management of inland waters (water supply, recreation, fishing, etc). HABs release toxic or chemical substances into the water which may be unpleasant (Jüttner, 1987) or lethal to all life forms (Gorham and Carmichael, 1988). The water quality problems caused by dense populations of cyanobacteria are intricate (Skulberg, 1996), and can have great health and economic impacts. As a consequence, the negative aspects of cyanobacteria have gained considerable research attention and public concern in recent years.

Although a number of countries have established research plans for Coastal HABs (CHABs), no similar plan exists for freshwater HABs (FHABs) till date. Eutrophication and FHABs can cause enormous remediation costs. Research is therefore needed to characterize occurrence and risks, and develop cost effective strategies for preventing,

suppressing and mitigating FHABs (Hudnell, 2010). The incidence of eutrophication in South East Asia has increased dramatically in recent years, coinciding with increases in loading from domestic and industrial effluents (Te and Gin, 2011). Singapore also harbors a lot of freshwater systems including reservoirs and lakes, and experiences nutrient loadings and anthropogenic inputs from wastewater effluents and storm water runoffs . These factors could contribute to emergence of FHABs.

However, there is a paucity of scientific data available addressing various issues related to FHABs. In order to implement selected strategies to prevent or suppress FHABs and mitigate the risks, policy determinations concerning the issuance of regulations or guidelines for FHABs and their toxins require sufficient information on: (1) the occurrence of blooms in freshwaters to determine if incidence warrants action; (2) dose-response relationships between toxin concentrations and adverse health effects and/or cell densities and ecological impacts to determine if the risks warrant action and; and (3) remediation methods to prevent, control and mitigate FHABs and predictive models to determine if cost effective means of reducing or eliminating the risks are available (Hudnell, 2010). The major challenge involves the assessment of toxins, especially cyanotoxins and their implications on release upon lysis of HABs.

2.2 Cyanobacterial Toxins

Cyanotoxins are a diverse group of natural toxins, which fall into three broad groups of chemical structure: (1) cyclic peptides, (2) alkaloids and (3) lipopolysaccharides (LPS). In the present study, cyclic peptides were only considered. Cyclic peptides consist of a group of hepatotoxins called microcystins (MCs) which are the most

commoncyanobacterial toxins found in the water systems (Carmichael, 1997). MCs are produced by at least six genera of cyanobacteria, including *Microcystis, Oscillatoria, Nostoc and Anabaena*. They are monocyclic heptapeptides containing an unusual amino acid (2S,

3S, 8S, 9S)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda) that is essential for the expression of biological activity (Dawson, 1998). Figure 2.1 shows the general structure of all MCs. The seven amino acids are numbered with variable portions shown as X, Z, R1 and R2. MCs are named using the one letter abbreviation for the amino acids substituted at the X and Z positions, respectively. The two MCs that are the subject of this doctoral study have different amino acids in the X and Z positions in Figure 2.1, but are otherwise identical [both R1 and R 2 are methyl groups]. For MCLR [neutral mass: 995.17] and MCRR [neutral mass: 1038.2], X is Leucine (L) and Arginine (R) respectively and Y position is Arginine (R) for both of them.

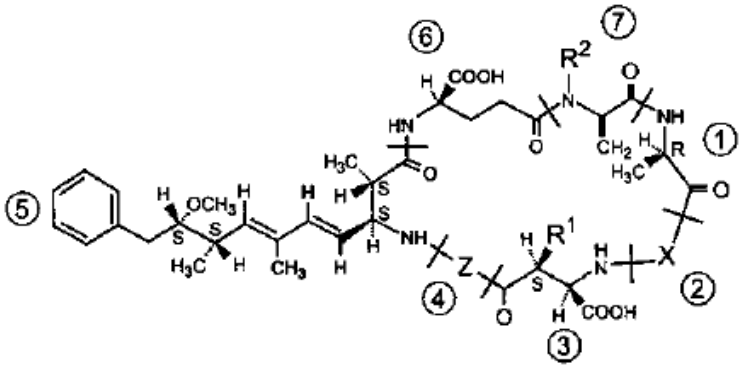


Figure 2.1: General Structure of MCs (Butler et al., 2009)

MCs are secondary metabolites which are produced intracellularly. Natural processes cause the cyanobacterial blooms to lyse, leading to the release of these toxins from the scum in the surface waters with adverse health effects on living biota (OECD, 2005).

2.3 Toxicology of MCs

The MCs that are widely studied are potent acute liver toxins with LD50 in mouse of about 50-500 µg kg^{-1} (intraperitoneal injection) (Carmichael, 1997). Early manifestations of liver damage include an increase in serum of liver enzymes, a sign of liver cell death, and increased liver weight. They also act as tumor promoters. The molecular basis of the tumor promotion is the inhibition of protein phosphatases 1 and 2A (PP-1 and PP-2A), the two key enzymes in cellular regulation (Carmichael, 1997; Yu, 1989, Harada, 1996). This enzyme removes phosphate from a protein, a common step in many biochemical pathways. This inhibition is believed to be a mechanism by which MCs destroy livers. Hepatocytes from animals treated with MCs appear to die by a process of programmed cell death, or cell suicide called apoptosis (Hooser, 2000). MCs appear to act similar to other akadaic acid promoters through PP-1 and PP-2A in contrast to phorbol esters which bind to and activate protein kinase C (Rapala et al., 1993). Mutagenicity has not been observed for purified toxins derived from Microcystis, but was clastogenic for humanlymphocytes (Chrous 1999).

In view of their toxicity and the worldwide occurrence of cyanobacterial blooms, MCs present a serious global health problem in water supplies, for both livestock and humans (Carmichael et al., 2001). The majority of reported MCs poisonings have occurred among domestic animals that drink freshwater containing cyanobacterial blooms (Briand et al.,

2003; Landsberg, 2002; Stewart et al., 2008; Schwimmer and Schwimmer, 1968). Thousands of livestock fatalities and numerous poisonings in dogs have been linked to the ingestion of cyanobacteria (Briand et al., 2003; Stewart, 2008). In North America, domestic animal poisonings have been linked to blooms of Microcystis sp. in California (DeVries et al.,1993), Colorado (Puschner et al., 1998), Georgia (Frazier et al., 1998), Michigan (Fitzgerald and Poppenga, 1993), and Mississippi (Kerr et al., 1987). Most of the poisonings were fatal and were associated with visible scum of cyanobacteria. Symptoms of MCs poisoning in domestic animals include diarrhea, vomiting, weakness and recumbency (Briand et al., 2003; DeVries et al, 1993). Although there have been no reports of human deaths occurring from the ingestion of MCs, there are numerous reports on a variety of health effects observed after exposure to MCs in drinking water, or from swimming in water in which cyanobacteria were present (Jochimsen et al., 1998). One of the earliest reports for human poisoning was from - Armidale, New South Wales, Australia where repeated blooms of *Microcystis aeruginosa* have been documented since the 1970's (Jochimsen et al., 1998). In 1981, an extensive bloom was associated with increased serum enzymes in tested individuals consistent with hepatotoxicity (Chorus and Bartram 1999). Another report from a place called Caruaru in Brazil, where 116 out of 131 patients experienced visual disturbances, nausea, vomiting, and muscle weakness following routine dialysis (Azevedo et al., 2002). One hundred of those affected by exposure to toxins then developed acute liver failure and 52 eventually died from symptoms of what is now called “Caruaru Syndrome” (Azevedo et al., 2002). The cause of this syndrome was determined to be cyanotoxins from reservoir water that had not been treated, filtered, or chlorinated before using it for

dialysis patients (Jochimsen et al.,1998). MCs were found in the water as well as the blood and livers of the patients. However, very little is known about the chronic low dose effects of MCs exposure. In China, it is reported that the highest incidence of liver cancer occurs in areas with abundant cyanobacteria in the surface waters (Chorus and Bartram , 1999).

Among 80 molecular variants of MCs isolated till date, MCLR, a hydrophobic variant, is considered to be the most commonly occurring and lethal toxin (WHO, 2003). A provisional safety guideline of 1.0 µg L^{-1} MCLR in drinking water was recommended by WHO (Falconer, 1999). Toxicity of MCLR based on intraperitoneal LD50 in laboratory mouse or rat injections was found to be 50 µg kg^{-1}. Another common variant known is MCRR which has a LD50 of 300 µg kg^{-1} (Carmichael, 1997). It can be seen that MCRR is less toxic than MCLR; however, both are released by same strains of cyanobacteria. They often occur together in a bloom episode. MCLR and MCRR are the two most common MCs encountered in HABs. In this study, MCLR and MCRR were used as model toxins.

2.4 Analytical approaches for MCs detection

In earlier studies, mouse bioassays (mostly using mice) were reported to be the only way to detect MCs. These assays are simple, but they are neither sensitive enough nor convenient from an ethical point of view (Falconer, 1993; Campbell et al., 1994). Although several invertebrates (including *Daphnia sp.*, *Drosophila melanogaster*, and mosquitoe larvae) have been investigated for their ability to detect the presence of MCs, none has been fully validated for use in routine monitoring.

Later, structural characterization and studies of the action mechanisms of the most widespread MCs allowed for the development of efficient methods such as enzyme-linked immunosorbent assay (ELISA) and protein phosphatise inhibition assay (PPIA). These bioassays resulted in low detection limits and high sensitivity. However, they lack specificity and hence cannot be used for routine measurements unless for specific purposes (Nagata et al., 1997; Pyo et al., 2005; Metcalf et al., 2001). The MC-dependent PPIA assay, for example, sometimes provides false positives, or does not allow detection of very low concentrations. Furthermore, false negatives may also occur using this mouse bioassay, as reported by Sim and Mudge (Sim and Mudge, 1993). However, the bioassays are very useful for evaluating water safety, as they show the overall toxicity of MCs. On the other hand, Fischer et al. (2000) developed an ELISA using polyclonal antibodies and reported limits of detection (LODs) below 1 μg L^{-1} in drinking waters, which is the value proposed by the WHO, without any sample preparation or preconcentration steps (Fischer et al., 2001). However, these immunoassays do not provide precise quantitative analysis of samples containing families of MCs with different levels of single toxins. ELISA can be used only as a screening assay eliminating negative samples for further chemical analysis. An ELISA developed for the detection of MCs using monoclonal antibodies reported variability of MC concentrations during eight months using the direct assay (Shen et al., 2003). Apart from bioassays involving animal testing and other immunoassays, there are physiochemical methods known as well now for detecting MCs.

High-performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC-MS) are the two main physiochemical methods used for detection of

MCs (Lawton et al., 1994b; Meriluoto et al., 1998; Bateman et al., 1995; Sano et al,,1992). These methods are mostly based on liquid-based separations combined with an ultraviolet (UV) or MS detector. The UV detector presents some disadvantages over the MS, because UV is not sufficiently sensitive and selective (many neutral interferences absorb in the same UV region as the MCs); moreover, the 80 MCs have very similar UV spectra. However, MS allows for identification of MCs and their degradation products in waters, and both sensitivity and selectivity can be improved using selected ion monitoring (SIM), selected reaction monitoring (SRM), or multiple reaction monitoring (MRM). The fragment ions observed for the MCs arise predominantly from the loss of water and then consecutive cleavages of the amide bonds that provide information concerning the amino-acid sequences. However, before any MC variant could be detected via UV/MS, it needs to be separated on a column. The separation of MC variants within a sample will be largely dependent on the composition of the mobile phase, and the stationary phase employed in the analysis. MCs can be separated using both gradient elution and isocratic mobile phases although the range of toxin variants that can be resolved when a gradient is employed is usually wider. One of the most commonly used HPLC methods involves separating the toxins on a C18 silica column using a gradient of water and acetonitrile, both containing 0.05% trifluoroacetic acid (TFA), which acts as an ion-pairing agent. This technique has been shown to separate up to 10 microcystins of varying polarity (Lawton et al., 1994b). However, it is usually necessary to analyze samples in parallel with toxin standards to accurately identify which variants are present (Lawton et al., 1994b).

Apart from HPLC and LC-MS, Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) has also been described for the detection of MCs in literature (Welker et al., 2002). MALDI-TOF-MS proved to be a reliable, rapid tool to detect and to identify MC variants using postsource-decay fragmentation. For monitoring MCs in environmental samples, MALDI-TOF-MS can provide considerable support to HPLC by identifying MC variants not available as purified standards. Another analytical platform tested for detection of MCs (particularly, MCLR) is Capillary electrophoresis (CE). It is not a commonly used method for the analysis of MCs because it requires sample preparation to achieve the required LOD (Limits of detection). In one work, CE-UV was used for the analysis of MCLR in waters (Gago-Martinez et al., 2003). CE was described as a powerful alternative for a fast and simple determination of the compounds, and the lack of sensitivity could be overcome by pre-concentration methodologies.

Before MCs can be detected through any of these platforms, sample pre-treatment followed by extraction and concentration is often required if they are in low concentrations. The sample pre-treatment of water samples containing MCs sometimes include a filtration step to separate the algal cell from the water in order to differentiate intracellular and extracellular concentration of MCs. Since this study is focused on extracellular MCs, further discussion on extraction and concentration would focus on extracellular MCs.

MCs in extracellular form are usually present in low concentrations except the immediate period after scum lyses, which makes their direct detection not feasible in most of the cases due to the inadequate sensitivity of analytical detection methods. Hence, pre-

concentration of water samples is usually performed using a number of methods prior to chemical analysis. For extracting dissolved MCs (extracellular MCs) from waters, after disruption of algal cells or from filtered waters, the most commonly used method is solid-phase extraction (SPE) and size exclusion chromatography (SEC). SPE and SEC are two suitable methods for the simultaneous extraction and clean-up from the aqueous samples. They are typically used to enrich environmental concentrations of MCs, or to eliminate other contaminants from complex samples such as animal and plant tissues followed by different detection modes (Hummert et al., 1999; Cong et al., 2006; Barco et al., 2002; Xu et al., 2008; Kwok et al., 2002).

Various kinds of packing materials and solvents have been tested for this purpose. However, the octadecyl silica (C18) in combination with methanol (or aqueous mixtures with methanol) is most frequently used, and has shown recoveries higher than 85% (Zhang et al., 2004; Harada et al., 2004; Hummert et al., 2001). A few studies used acidified solvents, (i.e. employing methanol with 0.1% trifluoroacetic acid (TFA)), resulting in satisfactory recoveries (Ortea et al., 2004). Rivasseau et al. (1998) showed that methanol–water (40:60, v: v) was suitable for the extraction of MCs by SPE C18 cartridges, although MCs showed increased solubility in methanol acidified with 1% TFA (Rivasseau et al., 1998).

However, these pre-concentration procedures are relatively cumbersome and time consuming. Moreover, some of these methods utilize toxic, hazardous and volatile organic solvents which are not environmental friendly.

Relatively newer techniques have been developed based on immunosorbents and molecularly imprinted polymers (MIPs) to improve the selectivity of the extraction and clean-up of water samples containing MCs (Krupadam et al., 2012). Aranda-Rodriguez et al. (2003) reported the comparison of the extraction and clean-up of six MCs between two different immunosorbents containing anti-MC-LR polyclonal antibodies and current SPE performed with a macroporous copolymer sorbent named HLB (hydrophilic–lipophilicbalanced) in cartridges. Recoveries for individual MCs with immunosorbents (>85%) were comparable to those obtained with SPE (>90%) (Aranda-Rodriguez et al., 2003). They also found that the immunosorbent extracts were free of interferences, and that enabled better detection and identification of MCs. In another work by Chianella et al., (2003), the extraction of MCLR with MIPs was reported, showing recoveries over 66% (Chianella et al., 2003). A recent study by Krupadam et al. (2012) also reported very high extraction efficiencies of MIP for MCLR as determined by a computer simulated model (Krupadam et al., 2012).

New biosensor and immunosensor technologies are also becoming available, with sufficient sensitivity and specificity to enable rapid 'on-site' screening without the need for sample processing. Ma et al. (2009) reported an immunosensor based on the relaxation of magnetic nanoparticles. The method was performed in one reaction and offered sensitive, fast detection of target toxin residues in water. The target analyte, MCLR competed with the antigens on the surface of the magnetic nanoparticles and then influenced the formation of aggregates of the magnetic nanoparticles. Accordingly, the magnetic relaxation time of the magnetic nanoparticles was changed under the effect of the target analyte. The use of this newly developed technique may find widespread

applications,especially for toxin residues sensing (Ma et al., 2009). Lawton et al. (2010) evaluated the sensitivity and cross reactivity of the commercially produced ImmunoStrip® and applied them to the detection of MCs in laboratory cultures and natural samples. It was observed that while the ImmunoStrip® was originally designed for the detection of MCs with a concentration of 10 mg L^{-1}, all 7 MCs that were tested could be detected below 1 mg L^{-1} (Lawton et al., 2010). Several advanced technologies are emerging for fast and on-site detection of MCs for routine monitoring. However, there is a need for demonstrating proper validation and local applicability of these developed technologies.

After extraction and concentration steps, samples are directed to any of the biochemical or physiological method for detection purposes. Selection of detection method would depend on a number of factors. Sample matrices, sensitivity and specificity required are some of the factors that determine the appropriateness of a particular detection technique for MCs. Conventional techniques may not work as efficiently and effectively when applied for routine purposes to a local water body. Consequently, techniques for detection/extraction should be adopted and developed based on the local context and relevance.

2.5 Interaction of MCs with aquatic life

Most organisms can come in contact with the released toxins, or with the cyanobacterial cells. This uptake by aquatic organisms suggests the possibility of the toxin accumulation in the aquatic food web. It has been shown that certain groups of organisms (e.g. protozoa, rotifers) are highly sensitive to these toxins which implies that

processes such as microbial loop are disturbed with negative consequences for otherorganisms in the food web (e.g. Fish, crustaceans) (Christoffersen, 1996; Nizan et al.,1986; Fulton and Pearl, 1987). MCs are toxic to fish at concentrations as low as a few micrograms per liter (μg L^{-1}), or possibly even fractional μg L^{-1} (Malbrouck and Kestemont, 2006; Ibelings and Havens, 2007; Wiegand and Pflugmacher, 2005). Considering that MCs have been frequently measured in waters with cyanobacterial blooms (WHO, 1999), it is not surprising that potential impacts on fish are receiving increased attention. Fish typically either ingest cyanobacteria, or their released MCs, or prey on organisms that have fed on cyanobacteria (Tencalla et al., 1994; Tencalla and Dietrich, 1997; Fischer et al., 2000). As with mammals, MCs are actively taken up by the liver in fish where they disrupt normal cellular activity by inhibiting protein phosphatases (Tencalla and Dietrich, 1997; Boaru et al., 2006). Inhibition of these enzymes in fish can ultimately result in widespread cellular death and loss of liver structure (Malbrouck and Kestemont, 2006). Protein phosphatases are particularly important during fish embryonic development because they regulate critical developmental processes (Gotz et al., 2000). Due to the limited capacity of fish to detoxify MCs, they easily succumb to the toxic effects of increased MC concentrations (Metcalf et al., 2000; Mohamed and Hussein 2006; Li et al., 2007). In the case of acute exposure, it could lead to fatal results while in the case of chronic exposure; they may face continual stress due to alteration at biochemical and metabolic levels. However, the impact of MCs on fish is often a complicated situation since massive fish kills following a bloom could not only be caused by MCs released from dying cells, but could also be due to the decreased oxygen and pH levels caused by the decaying bloom (Ibelings and

Havens, 2007). To understand the health impacts on aquatic organisms and design efficient prevention strategies, there is a need to study the toxic effects of MCs on fish systematically using controlled laboratory experiments with several different fish species and exposure routes. It is important to consider different routes of exposure to MCs such as oral, dermal etc. in accordance with the local context. Since Singapore has a tropical climate with no significant variation of climate parameters on a year to year basis, the climatic conditions remain favorable for the toxins to survive in the water phase for longer periods of time even after the bloom episode is over. Moreover, Singapore offers hot and humid climate which is favorable for growth of cyanobacteria resulting in algal outbreaks in fresh water reservoirs (Te and Jin, 2010). This favorable growth of cyanobacteria under tropical conditions makes it even more important to consider the fate and interactions of these released MCs on the immediate aquatic environment.

Data collected from earlier studies on the immediate (acute) lethality of MCs in fish have utilized IP injections of extracted MCs to determine the dose that is lethal to half the test population (LD50) (Malbrouck and Kestemont, 2006). The reported LD50 values of MCs in fish range from 20 to 1500 μg MCLR kg^{-1} body weight (Malbrouck and Kestemont, 2006). The large range of LD50 could reflect variation between fish species, or differences in toxin extraction, purification, or measurement methods. In general, mature fish are less sensitive to acute MCs toxicity than mammals (Malbrouck and Kestemont, 2006). However, IP injections of MCs are not analogous to field exposures since the toxin is absorbed faster and metabolized differently when administered into the abdominal cavity (as with the IP route) as compared to oral administration (Ibelings and Havens, 2007). In nature, fish are most likely subjected to sublethal impacts resulting

from exposure to MCs over days or weeks (i.e. chronic exposure to low levels of MCs).

Several studies have observed severe liver damage in fish following oral administration of MCs; usually in the form of freeze-dried cyanobacterial cells (Carbis et al., 1996, Malbrouck and Kestemont, 2006). The sublethal MCs concentrations shown below are commonly found in food items of fish during blooms. Apart from food, fish could also be exposed to extracellular MCs released in the water in balneation exposure (i.e. aquatic organisms being bathed in the water containing the trace levels of dissolved toxins). However, there are very few studies done on toxicological implications of extracellular MCs (Cazenave et al., 2006b, Jiang et al., 2011). Some of these studies have been discussed later in this chapter. Sublethal exposure is unlikely to cause lethality, but could alter the biochemical and metabolic pathways. These changes could be found among exposed fish groups, or could be passed to their next generations, both of which could be detrimental to the existing ecosystem balance.

In real aquatic ecosystems, organisms would often be exposed to trace levels of toxins persisting in water for long time periods following a lyses episode (Jones and Orr, 1994). To make a realistic evaluation of the toxicity of MCs, the selection of exposure route and the dosage are very important parameters for any experimental design (Ron et al., 2003). This toxicological evaluation would offer a better understanding in terms of bioaccumulation and uptake pathways involved in the toxicity of MCs. Organisms often adapt to the stress situations and respond differently in different conditions. Therefore, there is a great need to explore all the possible pathways for the evaluation of MCs toxicity for a complete, realistic risk assessment and risk management in water bodies.

Different fish species have been used in a large number of studies to evaluate the health impact of MCs toxicity. There have been a number of studies on *D. rerio* embryos, *C. paleatus, C. carpio, H. molitrix* and few other species either injected intraperitoneally with MCLR/MCRR or exposed to a cyanobacterial bloom. Biochemical, histopathological and behavioral patterns in the fish have been examined upon exposure to MCs, notably MCLR and MCRR (Wiegand et al., 1999; Cazenave et al., 2006a; Cazenave et al., 2006b; Blaha et al., 2004; Li et al., 2005). However, the uptake and severity of health effects vary largely depending upon the dose of these toxins and the exposure route. Most of the studies have reported biochemical enzymes such as glutathione peroxidase (GPx), glutathione-S-transferase (GST), glutathione reductase (GR) belonging to a class of antioxidants as study endpoints. The key antioxidants and the oxidative stress associated with them are reviewed in the following sections.

2.6 Oxidative stress caused by MCs

The term ‘‘oxidative stress”, refers to a cell’s state characterized by excessive production of reactive oxygen species (ROS) including hydroxyl radicals, superoxide anion radicals, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radicals and/or a reduction in antioxidant defenses responsible for their metabolism. Oxidative stress generates an imbalance between ROS production and its removal by the antioxidant defenses. Environmental pollutants such as MCs have the capability of generating ROS and disturbing the balance in otherwise healthy cells. MCs have also been reported to have the effect of generating oxidative stress in cells. The in vivo metabolism of these toxic compounds often leads to the formation of ROS, which significantly contribute to

their toxicity (Ding et al, 2001; Ding et al., 2000a and b; Li et al., 2003). The reduction products of molecular oxygen (superoxide radicals, hydrogen peroxide, hydroxyl radicals and hydroperoxyl radicals) is of great significance, as they may react with critical cellular macromolecules, possibly leading to enzyme inactivation, DNA damage, lipid peroxidation and ultimately cell death (Zegura et al., 2003 ; Zegura et al., 2008). Generation of ROS is an inevitable part of aerobic life. However, organisms have evolved with diverse mechanisms to combat the effect of normal flux of ROS generated by respiration, certain enzyme activities, and phagocytosis. These mechanisms include enzyme systems that act to remove ROS, low molecular weight compounds that directly scavenge ROS and proteins that sequester prooxidants. Together, they form the antioxidant defense systems (Hudson, 1990). Antioxidant defenses can be altered in response to oxidative stress, or increased ROS production in a variety of factors which could be intrinsic or extrinsic (Hudson, 1990). Instrinsic factors could be related to aging and disease while extrinsic factors could originate from environmental factors including pollutants and toxic chemicals. Environmental pollutants, such as heavy metals, are known to increase oxidative stress by increasing the load of ROS on cells which could alter antioxidant defense systems which are predominantly biochemical in nature. Alteration of biochemical defense systems is typically the initial response to any toxic insult and therefore their measurement can be sensitive indicators of altered cell function. These enzymes not only give us information about the state of the cell, but also serve as indicators of toxic effects caused by the contaminants at metabolic and molecular level. Consequently, the enzymes can serve as biomarkers of interest and can be used as a

component for risk assessment (Ron et al., 2003). Biomarkers and risk assessment would be discussed in later sections of this chapter.

Several research reports indicate that MCs can alter the antioxidant system and/or induce oxidative stress in diverse aquatic species and organs. MCs uptake has been related to the production of reactive oxygen species such as free radicals (Ding et al., 2000a, 2001; Li et al., 2003), leading to an increase in lipid peroxidation (Pinho et al., 2005; Jos et al., 2005; Prieto et al., 2007), DNA damage (Zegura et al., 2003, 2008; Votto et al., 2007), DNA–protein crosslink (Leão et al., 2008), mitochondrial damage (Ding and Ong, 2003) and alteration of the antioxidant defense system (Vinagre et al., 2003; Pinho et al., 2005; Cazenave et al., 2006a,b; Prieto et al., 2007; Amado et al., 2009). However, the causes and the mechanisms involved in these responses are not completely understood. Table 2.1 lists out the various studies conducted at different time points among fish to evaluate oxidative stress produced by exposure to MCs. Along with the increased levels of ROS production, several studies have provided evidence in favor of the alteration in GSH concentration due to MCs exposure (Ding et al., 2000b). GSH is the most abundant antioxidant, non-protein thiol, being found at the millimolar range in most cells (Dickinson and Forman, 2002; Maher, 2005). It was observed that following an exposure, there was an initial increase of intracellular GSH which is probably due to its conjugation with MCs, as shown by Pflugmacher et al. (1998), triggering the synthesis of new GSH. Pflugmacher et al. (1998) showed that GSH conjugates with MCs to form soluble glutathione-MCs conjugate which can be easily excreted, thus protecting the organism from its MC bioaccumulation (Pflugmacher et al., 1998). However, on continued exposure to MCs, there was a subsequent GSH depletion, which was considered to be

related to cell membrane damage and consequent GSH efflux (Ding et al., 2000b) since MCs are incorporated into the cell via OATPs (Organic anion transporting polypeptide) which uses GSH as driving force for the exchange with MCs (Figure 2.2). In this way, MCs incorporation by itself could induce efflux of intracellular GSH reducing the detoxification capability of the cell. MCs were also suggested to disrupt the mitochondrial electron transport chain (ETC), thus favoring ROS generation (Ding et al., 2002). Mitochondria are not only the main site of production of ROS, but also the main target of such toxic molecules, so the maintenance of its antioxidant capacity is vital for cell integrity (Aon et al., 2007). It has been evaluated that mitochondria do not have the enzymatic pool associated with GSH synthesis and mitochondrial GSH is of cytoplasmatic origin (Meister, 1995). Therefore, depletion of cytosol GSH could reflect in a decreased GSH concentration inside mitochondria, a situation that favors the described increase in ROS production and ETC disruption induced by MCs. The alteration in GSH concentration can also have effects in several signaling pathways that are modulated by alterations in the redox status of the cell. Dickinson and Forman (2002) hypothesized that many environmental agents exert their deleterious effects by altering, either directly or indirectly, the cellular redox status through manipulation of thiols metabolism such as GSH (Dickinson and Forman,2002). There is another explanation for the increase in production of ROS by MCs. MCLR has been shown to bind the beta subunit of ATP-synthase, which can contribute to the intensification of the mitochondrial membrane depolarization, disruption of ETC and ROS generation (Zhang et al., 2006, 2007, 2008). Mitochondrial membrane depolarization leads to the release of cytochrome c, signaling to apoptosis (Figure 2.2), an effect of MCs that has been shown in severa

studies using cell lines from different organisms, including fish (Ding et al., 2002; Zhang et al., 2006, 2007, 2008).

It is clear from the above discussion that MCs generate oxidative stress mainly by inducing an increase in ROS production associated with a depletion of antioxidant defenses. Besides all these effects, MCs are also known to inhibit protein phosphatases, leading to a cellular hyperphosphorylation state, together with the pro-oxidative environment inside the cell would lead to Nrf2 (Nuclear factor (erythroid-derived 2)-like 2; a transcription factor that induces the expression of various genes for antioxidant enzymes) migration in to the nuclei, promoting the transcription of genes involved in antioxidant response, such as GST, GPx and GR (Kobayashi et al., 2009). Phosphorylation and ATP (Adenosine Triphosphate) leads to favoring of continuous depletion of GSH; phosphorylation activates GST and GPx. All these events together cause alteration in redox status of the cell and mitochondrial disruption what could lead to the release of cytochrome c which, in turn, activates signaling cascades to apoptosis.

These biochemical parameters including antioxidant enzymes, though highly sensitive, are not selective and specific to the kind of toxicity faced by the organism. Activities of these biochemical parameters namely, enzymes, vary considerably depending upon the dosage and time of exposure to the concerned organism. Moreover, continuous exposure to low levels of toxins can confer resistance to the organism and subsequently, they can adapt their systems to this oxidative stress observed by a variety of ways (Koyeli et al., 2005). However, adaptive response has not been studied for low doses of MCs. Since it is known that the biochemical systems are affected in an event of acute MCs exposure, it

is highly relevant and justifiable to study the changes in the antioxidant enzyme systems at various time points in an organism on exposure to extracellular low doses of MCs. It would also help in evaluating biomarkers which could serve to biomonitor for risk management of water bodies with local relevance.

Table 2.1

Evidences of oxidative stress on aquatic organisms exposed to MCs

(Amado and Monserrat, 2010)

Species	Organ	Exposure	Antioxidant response analyzed	Oxidative stress parameters analyzed	Main observed effects	Reference
Laeonereis acuta (P)	Whole animal	Immersion in *Microcystis aeruginosa* extract (~2 μg MIC/mL) during 48 h	CAT, GST and GR activities	LPO	Lower CAT activity and no alteration on GST activity; higher LPO levels and DNA–protein cross-links content	Leão et al. (2008)
Chasmagnathus granulatus[a] (C)	Gills (anterior and posterior)	Injected with *Microcystis aeruginosa* extract (39.2 μg MIC/L) during 48 h	Total antioxidant capacity (TOSC assay[b]) and GST activity.	LPO	Higher TOSC and GST in posterior gills.	Vinagre et al. (2003)
Chasmagnathus granulatus[a] (C)	Hepatopancreas	Injected with *Microcystis aeruginosa* extract (17.6 μg MIC/L) during 72 h and one week of exposure	CAT, SOD and GST activities	LPO	Higher CAT and GST activities in crabs exposed for 7 days. No effect on SOD activity and LPO levels	Pinho et al. (2003)
Chasmagnathus granulatus[a] (C)	Hepatopancreas	Forced ingestion (~1 and 5 μg MIC/kg) during 168 h	CAT, GST and SOD activities	LPO and protein carbonyl groups	Biphasic alteration of CAT; Augmented GST activity; Higher LPO levels	Pinho et al. (2005)
Chasmagnathus granulatus[a] (C)	Hepatopancreas	Gavage with *Microcystis aeruginosa* extracts (34, 172, 860 μg MIC/kg) during 6, 12 and 72 h.	GST activity	LPO	Higher GST activity in crabs exposed to 860 μg MIC/kg for 12 h Higher LPO levels in crabs exposed to all doses after 72 h of exposure	Dewes et al. (2006)
Danio rerio (F)	Fish embryos	Immersion in REKO medium containing 0.1, 0.5, 1, 2 and 5 μg MIC-LR/L over ontogenetic development and after hatch (3 and 5 days)	sGST, mGST and GPx activity	None	Dose dependent increase in sGST and mGST activity over ontogenetic development (from 0.1 to 2 μg MIC-LR/L). The higher dose suppressed soluble GST activity. GPx increased in 0.5 μg MIC-LR/L exposure dose	Wiegand et al. (1999)
Hypophthalmichthys molitrix (F)	Hepatopancreas	Immersion in a cyanobacterial bloom (*Microcystis ichthyoblabe* 60% and *M. aeruginosa* 40%, 4–116 × 10^6 cells/mL	GSH	LPO	Higher GSH and no LPO effect	Bláha et al. (2004)
Oreochromis sp (F)	Liver, kidney and gills	Cyanobacterial cells mixed with a commercial fish food or crushed into a commercial fish food through 14 and 21 days.	SOD, CAT, GPx, GR activities	LPO	In general, antioxidant enzymes and LPO were increased after 21 days of exposure to crushed food in almost all organs, but liver was the most affected.	Jos et al. (2005)
Misgurnus mizoleps (F)	Liver	Orally exposed to 75 mg of dry cells/kg body weight (equal to 10 μg MIC-RR/kg body mass), for 28 days	SOD, CAT, GPx activities	LPO	Activity of antioxidant enzymes was increased and LPO remained stable.	Li et al. (2005)
Danio rerio (F)	Fish embryos	Immersion in REKO medium with 25 μg MIC-RR/L or 25 μg MIC-LF/L during 24 h	sGST, mGST, CAT, POD, GPx, GR	None	Higher sGST, mGST and CAT activity; no effect in the other analyzed enzymes	Cazenave et al. (2006a)
Corydoras paleatus (F)	Liver, gill, intestine and brain	MIC-RR dissolved in water (up to 1 μg/L) during 24 h	CAT, GPx, GR, GST, POD activities	TBARS	Decreased GST activity in all organs; the other antioxidant enzymes augmented in liver; higher LPO levels in brain	Cazenave et al. (2006b)
Oreochromis sp (F)	Liver, kidney and gill	I.p. injection of a single dose of 500 μg MIC-LR/kg or 500 μg MIC-RR/kg and killed after 7 days.	SOD, CAT, GPx; GR	LPO	In general, antioxidant enzymes, mainly SOD and CAT, and LPO levels were increased	Prieto et al. (2006)
Oreochromis niloticus (F)	Liver, kidney and gills	Food pellets with 1350 μg/g of pellet during 24 h	CAT, GPx, GR and SOD activities	LPO and protein carbonyl groups	In general, a decreased activity of antioxidant enzymes; higher LPO levels in all organs. Higher protein oxidation in liver.	Prieto et al. (2007)
Tinca tinca (F)	Liver and kidney	Orally exposed to cyanobacterial cells dosing 5, 11, 25 and 55 mg MC-LR/fish mixed with the food	SOD, CAT, GSH, GSH/GSSG	LPO and protein carbonyl groups	SOD activity decreased in a dose dependent manner in liver and kidney; decrease in CAT activity in the liver in the two higher doses and no effect in CAT activity in kidney; no effect on GSH and GSH/GSSG levels; LPO increased in the two higher doses; no effect in protein oxidation	Atencio et al. (2008)
Carassius auratus (F)	Liver, kidney and intestine	One injection (50 and 200 μg MIC/kg) and then followed up to 168 h	Expression of several GST genes	None	In general decreased transcription of several GST isoforms (including α, π and θ)	Li et al. (2008a,b)
Jenynsia multidentata (F)	Liver and brain	Food pellets with MIC-RR (up to 1 μg/g of pellet) and analyzed during 24 h	GST activity	None	Higher GST activity at the highest dose	Cazenave et al. (2008)
Jenynsia multidentata (F)	Liver, brain, gills and muscle	*Microcystis aeruginosa* cells (RST9501) extracts dissolved in water to reach 5 and 100 μg/L toxin	Total antioxidant competence against peroxyl radicals (ANCOMROS)	Protein oxidation (carbonyl groups)	Increased ANCOMROS in liver and decreased antioxidant capacity in brain and gills. There was no protein oxidation in liver.	Amado et al. (2009)

CAT: catalase. GPx: glutathione peroxidase. GST: glutathione-S-transferase. sGST: soluble glutathione-S-transferase. mGST: microssomal glutathione-S-transferase. LPO: lipid peroxidation. MIC: microcystin. MIC-RR: microcystin RR isoform. MIC-LF: microcystin LF isoform. MIC-LR: microcystin LR isoform. POD: guaiacol peroxidase. SOD: superoxide dismutase. TOSC: total oxyradical scavenging capacity. P, C and F refer to polychaeta, crustacean and fish species, respectively.

[a] Now cited as *Neohelice granulata*.

[b] Winston et al. (1998).

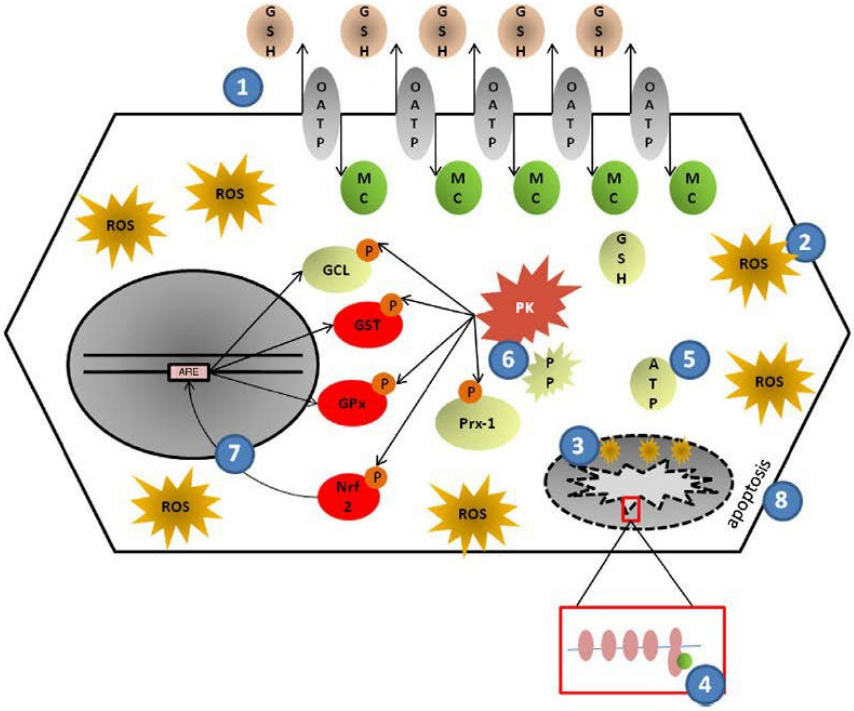


Figure 2.2: Hypothetical model of MCs induced toxicity based on literature data. OATP: organic anion transporting polypeptide; PK: protein kinases; GCL: glutamate cysteine ligase; ARE: antioxidant responsive element (Amado and Monserrat, 2010).

2.7 Biomarkers

Biomarkers are indicators that are present in body fluids, cells or tissues reflecting biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response (NRC, 1987). Toxicological effects at higher hierarchical levels are always preceded by earlier changes in biological processes (Figure 2.3), allowing the development of early-warning biomarker signals (Bayne et al., 1985). In an environmental context, biomarkers serve as sensitive indicators demonstrating that (a)

toxicants have entered organisms, (b) have been distributed between tissues, and (c) are eliciting a toxic effect at critical targets (McCarthy and Shugart, 1990). Biomarkers may provide important insights into the potential mechanisms of contaminant effects. For example, by screening multiple biomarker responses, important information can be obtained about organism toxicant exposure and stress. A pollutant stress situation i.e. a situation where high pollutant loading occurs in the ecosystems normally triggers a cascade of biological responses, each of which can, in theory, serve as a biomarker (McCarthy et al., 1991). Biomarkers can be used for risk assessment and risk management planning. They can also serve as screening tools for any contingency plans laid out to prevent an alarming situation from arising, for example, HABs. Details on risk assessment and management are given in the following sections.

For environmental contaminants, lot of animal species have been considered and used for assessing biochemical and toxicological endpoints. However, not all these species used for toxicological assessment can be used for monitoring as, biomonitoring can possibly be done with species which are ubiquitous in nature and are easy to maintain rear and handle. Fish species are easy to maintain and handle. Thus, out of all the other species, fish have always attracted considerable interest in studies, aimed at assessing biological and biochemical responses to environmental contaminants (Powers, 1989). Fish can be found virtually everywhere in the aquatic environment and play a major ecological role in the aquatic food-webs because of their function as a carrier of energy from lower to higher trophic levels (Beyer et al., 1996). Therefore, the understanding of toxicant uptake, behavior and responses in fish may, therefore, have a high ecological relevance. Most of the general biomarker criteria appear to be directly transferable to certain fish biomarkers

(Stegeman et al., 1992). Fish are generally considered to be the most feasible organisms for pollution monitoring in aquatic systems since it is possible to determine the biomarkers of interest and assess aquatic exposure using fish species. Biomarkers of effect include the measurable biochemical, physiological or other alterations within tissues or body fluids of an organism that can be recognized as associated with an established or possible health impairment or disease. Biomarkers of exposure indicates the inherent or acquired ability of an organism to respond to the challenge of exposure to a specific toxic substance, including genetic factors and changes in receptors which alter the susceptibility of an organism to that exposure (Ron et al., 2003). Figure 2.4 lists the principal scheme of responses in fish to the detrimental effects of pollutant exposure. Above a certain threshold (in pollutant dose or exposure time), the pollutant-responsive biomarker signals deviate from the normal range that is observed in unstressed conditions, finally leading to the manifestation of multiple effects at higher hierarchical levels of biological organization.

Biomarkers can be categorized in a variety of ways depending upon whether the study is aimed at exposure assessment, or effect assessment. However, it is important to include different levels in the hierarchy- from molecular to the ecosystem level to ensure efficient and effective risk management.

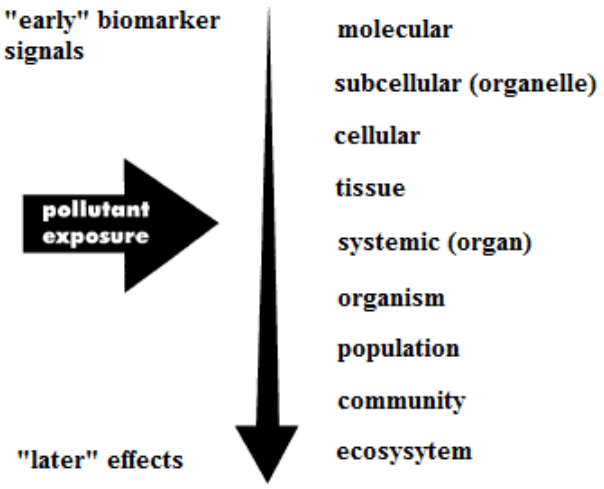


Figure 2.3: Schematic representation of the sequential order of responses to pollutant stress within a biological system. Taken from Bayne et al., (1985).

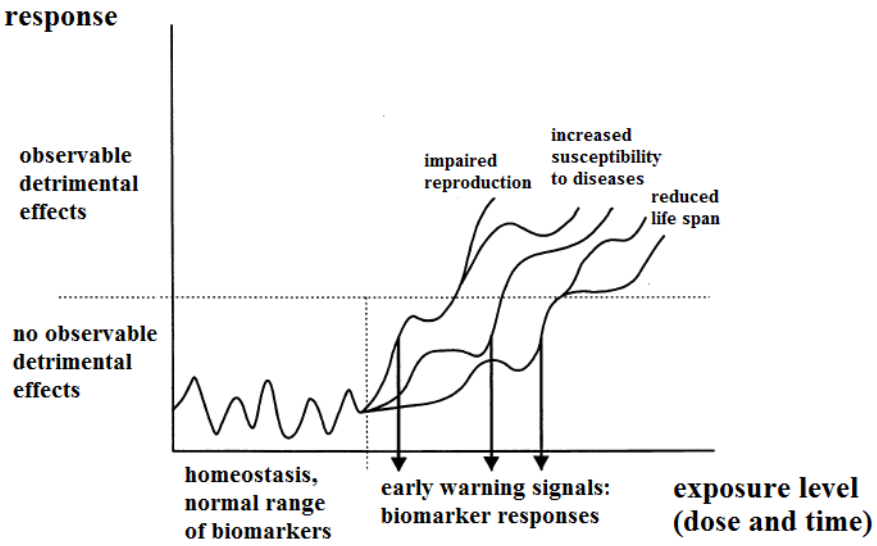


Figure 2.4: The principal scheme of responses in fish to the detrimental effects of pollutant exposure. Adapted from McCarthy et al. (1991).

In the current study, biochemical, histological, metabolic and gene biomarkers were evaluated and studied. They are in the same order of relevance. These biomarkers were selected based on the order of importance and the concerned toxicant studied.

2.7.1 Biochemical markers:

Oxidative stress parameters are usually studied for biochemical markers. Oxidative stress markers are the initial line of defense response by any organism under environmental stress. Many environmental contaminants (or their metabolites) have been shown to exert toxic effects related to oxidative stress (Winston and Di Giulio, 1991). Oxygen toxicity often leads to injurious effects among organisms due to ROS and oxygen free radicals or oxyradicals as previously described in other sections elsewhere in this chapter (Di Giulio et al., 1989). The activities of the antioxidant enzymes, which defend the organisms against ROS, are critically important in the detoxification of radicals to non-reactive molecules. Antioxidant enzymes can form an important biomarker as they are very sensitive. However, these antioxidant enzymes usually form a non-specific response and hence there is a need to explore further for biomarkers specific to a particular contaminant. Nevertheless, it is a reliable screening measure which could then further lead to in-depth studies.

2.7.2 Histological markers:

Determination of adverse effect resulting from toxic exposures can be performed histopathologically, by investigating lesions, alterations or tumor formation (neoplasms) in fish tissues, or by simply observing the accumulation/localization of the contaminant of interest in fish tissues. Histopathological studies cannot serve as a sole biomarker on

its own, but it is often useful for supporting the data obtained from other biomarkers and can help in its validation. Histopathology has received lot of attention in the case of MCs toxicity since MCs are known to be hepatoxins and tumor promoters, uptake and toxicokinetic effects can be easily observed and studied microscopically (Kotak et al., 1996; Tencalla and Dietrich, 1997). Apart from pathophysiology related to MCs and their intake, researchers have also reported the localization of MCs using histological studies. Immunohistochemistry (IHC) is the procedure used by the researchers to qualitatively study the biodistribution and localization of MCs (Djediat et al., 2010, Yoshida et al., 1998). IHC refers to the process of detecting antigens (e.g. proteins) in cells or tissues section by exploiting the principle of antibodies binding specifically to antigens in tissues. There are commercial antibodies available for MCs that target the ADDA moiety in MCs (common to all MCs), which could be used to study the immunolocalization. Several authors have reported the use of these antibodies for different organisms in specific fish species (Djediat et al., 2010; Yoshida et al., 1998; Guzman and Solter, 2002; Lance et al., 2010) to study the biodistribution of MCs after exposure. Although the results in various tissues cannot be quantified, they could still be used to complement the data or information obtained from other biological platforms. Hence, it would serve as biomarkers of exposure (bioaccumulation markers).

2.7.3 Metabolic markers:

MCs toxicity is mainly exhibited by its inhibition of an important class of enzymes known as phosphatases present in living tissues and organs (described earlier in section 1.3 of this chapter). These enzymes remove phosphate moiety from its substrate by

hydrolyzing phosphoric acid monoesters into phosphate ion and a free hydroxyl group (Carmichael, 1997; Yu, 1989, Harada, 1996). The removal of a phosphate group may activate or de-activate an enzyme, or enable a protein-protein interaction to occur (therefore protein phosphatases are integral to many signal transduction pathways). Protein Phosphatases also play a crucial role in biological functions and controls nearly every cellular process, including metabolism, gene transcription and translation, cell-cycle progression, cytoskeletal rearrangement, protein-protein interactions, protein stability, cell movement, and apoptosis (Pallen et al., 1992). Inhibition of these enzymes by MCs could disturb the cellular processes and metabolic pathways. The perturbation could be monitored from the efflux and influx of metabolites through these biochemical pathways. Perturbed biochemical pathways could, in turn, result in metabolic markers which are specific to MCs toxicity. These metabolic markers gain importance in the case of chronic and sub- chronic toxicity where lethality is not observed. However, there are still changes occurring at biochemical and metabolic levels. Studying these metabolic markers could also offer great insights into additional mechanistic details pertaining to MCs toxicity. Current science and technology offers lot of high-throughput analytical platforms in terms of detection and separation techniques which could target small molecules (metabolites) in the tissues, body fluids and cells (Dunn and Ellis, 2005). The study of metabolites is often referred as metabolomics. The metabolome often represents the collection of all metabolites in a biological cell, tissue, organ or organism, which are the end products of cellular processes. In the context of MCs, metabolomic investigations are still in its infancy. Research inputs are required in this area, from different organisms exposed to different set of environmental conditions, to build a

strong database of metabolic markers which could then be used for further investigations and risk management systems. Since metabolic markers are very specific to a particular contaminant in question, they could play a very important role in biomonitoring. Exploratory and preliminary studies are required to be conducted in this current unexplored area for gaining better scientific insights into toxicological implications from extracellular MCs exposure.

2.7.4 Transcriptomic/Genetic markers:

The exposure of an organism to toxicants may induce a cascade of events (Shugart et al., 1992), including formation of structural alterations in DNA, procession of DNA damage and subsequent expression in mutant gene products, and diseases (e.g. cancer) resulting from the genetic damage. The detection and quantification of various events in this sequence may be employed as biomarkers in organisms exposed to toxic substances such as MCs in the environment. Gene expression has been studied in a variety of environmental contaminants across the species. However, for MCs, research with a focus on gene expression remains unexplored. Very little is known about the changes in fish, including the regulation of the transcriptional program of fish tissues in response to MCLR or MCRR exposure. Earlier investigations were restricted to the gene expression profiles for antioxidant enzymes, heat shock proteins (in *Synechocystis sp*.) and apoptotic pathways (Li et al., 2009; Brzuzan et al., 2009). Recently, investigators have applied gene expression analyses in an effort to better characterize biochemical pathways influenced by MCs, specifically MCLR in fish. Gene expression studies were done with adult zebrafish injected (intraperitoneal) with MCLR (Wei et al., 2008). The results

revealed that numerous immune-related genes, in addition to genes involved in tumorigenesis and cell cycling, were differentially regulated in liver tissue (Wei et al., 2008). In larval zebrafish, immune-related genes and heat-shock proteins were also differentially expressed in targeted analyses as assessed by quantitative PCR (Li et al., 2009). Similar results were reported by Wei et al., (2008). Their study also confirmed that MCLR mainly influenced the cell cycle and mitogen-activated protein kinases (MAPK) signaling pathways. In addition, many immune-related genes were also influenced. They suggested that MCLR could promote tumorigenesis and cause immunotoxicity in fish (Wei et al., 2008). These studies revealed other mechanistic aspects of MCLR toxicity which were not known to scientific community earlier. Rogers et al. (2011) also reported up-regulation of vitellogenin genes (*vtg*) (19.2-fold to >100-fold on arrays) in *microcystis*-exposed larvae. Up-regulation of *vtg* indicates exposure to estrogenic substances and suggests that *microcystis* may be a natural source of environmental estrogens (Rogers et al., 2011). Global gene expression analysis could confer better insights and could reveal lot of new details about the mechanism of toxicity. These could serve as highly sensitive and specific biomarkers of exposure and consequently could enrich the database of biological markers pertaining to MCs toxicity. Efforts in this area could lead to development of better and more effective risk management strategies.

Identification of biomarkers may serve as a useful tool in several steps of the risk assessment process: effect, exposure and hazard assessment, risk characterization or classification, and monitoring the environmental quality of aquatic ecosystems.

2.7.5 Risk Assessment:

Risk assessment (RA) can be defined as the process of assessing magnitudes and probabilities to the adverse effects of human activities or natural catastrophes (Suter, 1993). Risk assessment consists of eight steps: Hazard identification, Effect assessment, Exposure assessment, Risk characterization, Risk classification, Risk-benefit analysis, Risk reduction and monitoring. Detailing of these steps is out of scope for this study. This study primarily focuses on indentifying "biomarkers" which could assist further in risk assessment. This doctoral study aims at the first three steps of risk assessment process, namely, hazard identification (detecting MCs in local water systems), exposure assessment (biodistribution through histological markers) and effect assessment (biochemical, metabolic and molecular markers). Since this study focuses on extracellular MCs, for the next level of steps in risk assessment process, inputs are required from other exposure routes as well for complete risk assessment of a water body following systems approach. This could further aid in environmental monitoring (Figure 2.5).

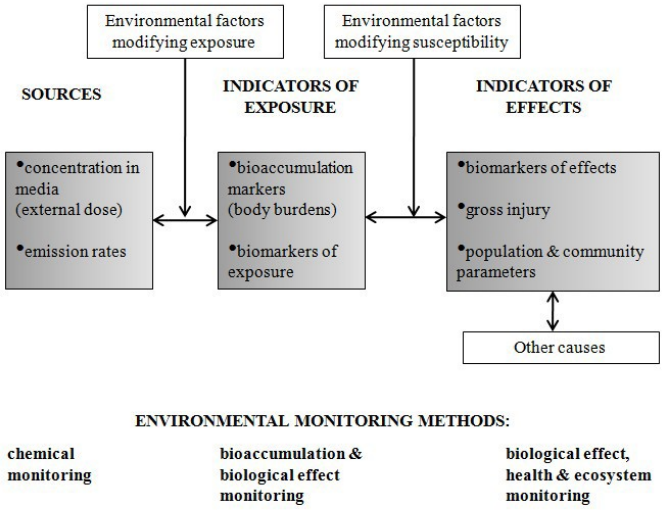


Figure 2.5: The relationship among the components of the risk assessment and characterization stage including their respective environmental monitoring methods (Ron et al., 2003)

A thorough understanding of the relationships between biomarker responses and survival, growth or reproduction is generally considered to be a prerequisite for the use of biomarkers in RA (Van Gastel and Van Brummelen, 1996). It is now acknowledged that biomonitoring is necessary for a reliable RA.

This study aims at identifying certain biomarkers that are specific to toxicity on zebrafish organs due to extracellular MCLR and MCRR. The identified biomarkers could further be used for environmental monitoring, thus filling the knowledge gaps that are exist today for toxicity of extracellular MCs.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Experimental Design

3.1.1 General Work Flow

The general work flow employed in this doctoral study is shown in Figure 3.1. The study has been divided into two broad components: analytical method development and toxicological investigations. Under the analytical component, extraction and detection of extracellular MCs (MCLR/MCRR) using a novel solvent was carried out. The method developed for quantitative measurement of MCs was validated with field samples. Under the toxicological component, impacts of extracellular MCLR and MCRR on aquatic health were studied using *Danio rerio* (zebrafish) as a model organism. The concentrations of MCs (0.1- 10 µg L^{-1}) for exposure assessment were selected based on their local environmental relevance and the literature. The overall aim was to identify potential biomarkers through the use of various platforms in toxicological experiments.

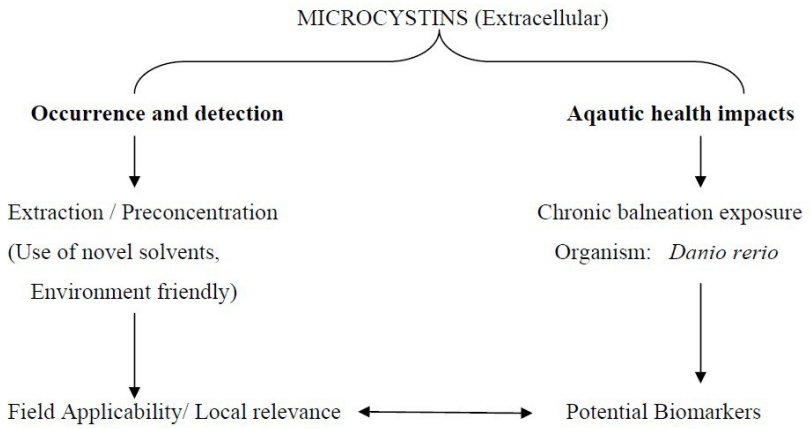


Figure 3.1: General work flow for the study

3.2 Chemicals and Reagents

3.2.1 Analytical Standards

Standards of MCLR and MCRR were obtained from Alexis Biochemicals (Switzerland). Internal standards for metabolomic studies (polar metabolites): DL-2-aminobutyric acid, 1-naphthylamine, 2-aminoanthracene, 2,3,4,5,6-pentafluorobenzoic acid were purchased from Sigma Aldrich Corporation (U.S.A).

3.2.2 Other Chemicals / Reagents / Antibody

Methanol (HPLC grade), 100% ethanol (molecular grade), chloroform (HPLC grade), tetrahydrofuran (HPLC grade), 1-butyl-3-methylimidazolium hexafluorophosphate (BMiM PF6) and formic acid were obtained from Merck (Germany). Milli-Q water (18 MΩ) used in all experiments was obtained through a Milli-Q (Millipore, Bedford, MA) water purification system. Potassium hydrogen phosphate and potassium dihydrogen phosphate were purchased from BDH (VWR, Singapore). EDTA (Ethylenediaminetetraaceticacid), Paraplast (paraffin wax), 10% formalin solution, goat serum, hydrogen peroxide (H2O2), DPX moutant, isopropyl alcohol, Diethylpyrocarbonate (DEPC)- treated water, RNA-ase free water were purchased from Sigma Aldrich Corporation (U.S.A.). A general monoclonal antibody to MCs (ADDA specific; AD4G2) was purchased from Alexis Biochemicals (Switzerland). Trizol reagent® was purchased from Life technologies, (U.S.A). Phosphate buffered saline with tween (PBST, 20 X) was purchased from Cell Signalling Technology (U.S.A.).

3.3 Experimental Workflow

3.3.1 Analytical studies

For analytical studies, 1-butyl-3-methylimidazolium hexafluorophosphate (BMiM PF_6) was used to extract and preconcentrate MCLR and MCRR. Details about the extraction procedure are given elsewhere in section 3.5.1.

3.3.2 Toxicological Studies

Toxicological components of this study are described in Figure 3.2. The potential toxic effects of extracellular MCLR and MCRR were evaluated at molecular, cellular, biochemical and histological levels. In biochemical studies, antioxidant enzyme systems (namely GPx, GST, GR and SOD activities) were evaluated in zebrafish organs (gills, intestine, liver, brain). Under cellular and molecular levels, metabolites and gene markers were evaluated, respectively in zebrafish organs (gills, brain, liver and intestine) after exposure to extracellular MCLR/MCRR. Under histological levels, markers for bioaccumulation of MCLR and MCRR were evaluated.

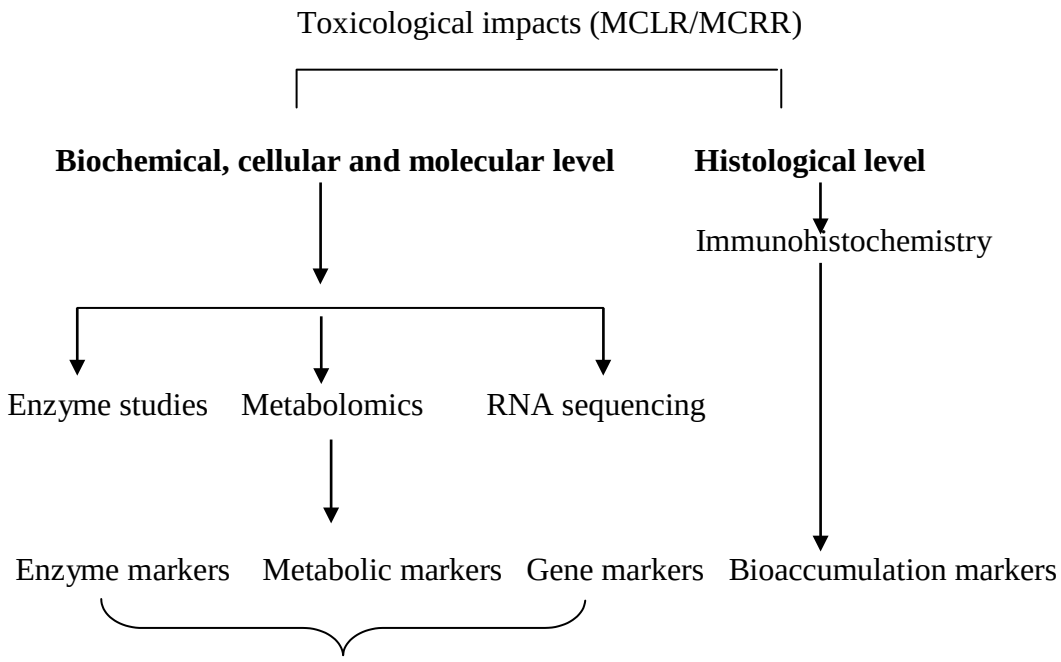


Figure 3.2: Work flow and components of toxicological studies.

3.3.3 Experimental Design for biochemical studies

Experimental design and workflow for fish exposure studies are given in Figure 3.3. The exposure of MCLR and MCRR to zebrafish is a part of toxicological impact assessment studies. Fish exposure was done as a preliminary study to evaluate dose response of extracellular MCLR and MCRR on antioxidant enzyme systems (biochemical studies) of zebrafish.

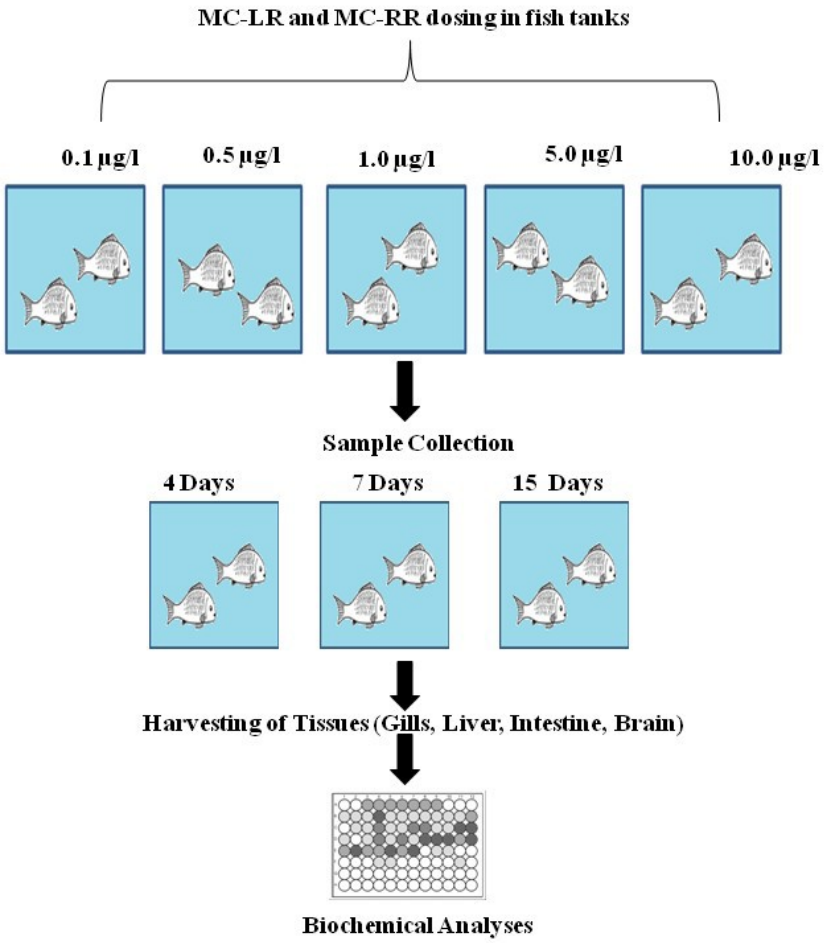


Figure 3.3: Work flow for exposure experiments (Biochemical studies)

3.3.4 Experimental design for toxicological investigations

Experimental design and workflow for fish exposure are shown in Figure 3.4. The exposure of MCLR and MCRR to zebrafish is a part of toxicological impact assessment studies. Zebrafish exposure was done to evaluate the impact of extracellular MCs (under chronic conditions) at cellular, histological and molecular levels. The dose of exposure was determined by the outcome of previous preliminary exposure studies. The highest dose from the first set was taken for this exposure.

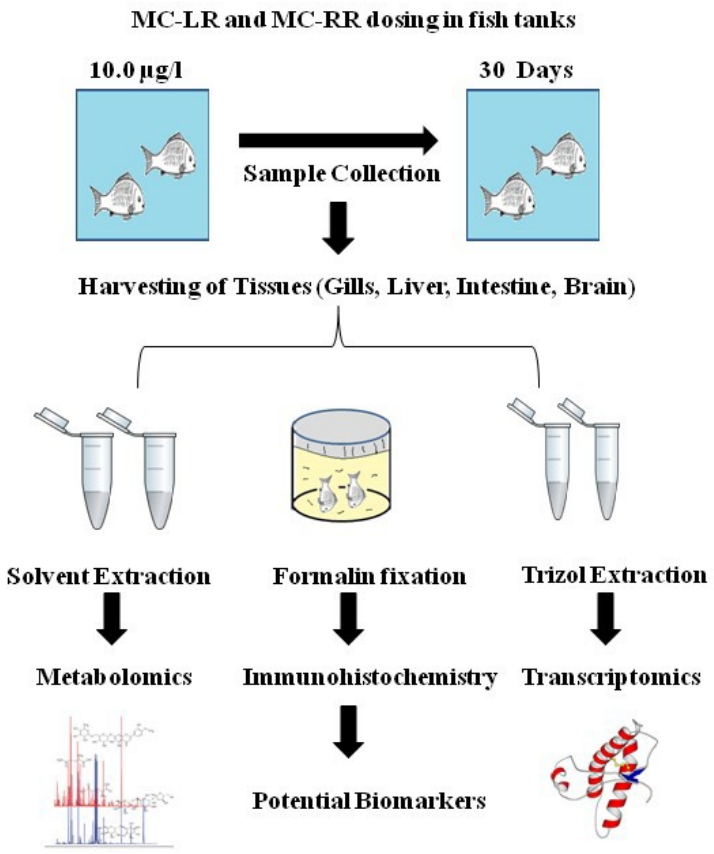


Figure 3.4: Work flow for exposure experiments (Toxicological investigations)

3.4 Model organism

3.4.1 Selection

Zebrafish (adult), a tropical freshwater fish, were selected in this study for several reasons. First, zebrafish represent an important vertebrate model organism that is widely used in scientific research (Hill et al., 2005). Second, earlier toxicological reports have been mainly focused on early life stages of zebra fish with little or no attention on adult fish (Cazenave et al., 2006a; Wiegand et al., 1999). Last, it is easy to maintain zebrafish

in the laboratory conditions since they are small, and can be maintained at low husbandry cost in large numbers.

3.4.2 Procurement and maintenance

Adult (male) zebrafish were procured from the mainland tropical fish farm, Singapore. The fish were transported to laboratory with closed water tanks. The fish obtained were of the same age (3 months) and were reared under identical conditions. The pure breed for spawning was maintained by the farm. Regular quality checks for water quality, oxygen levels and temperature were done in the farm for quality assurance. The fish were fed once a day with commercial fish food.

Fish, received from the local farm, were maintained in plastic disposable tanks, and were then acclimated to the local laboratory conditions during 10 days prior to conducting experiments. They were maintained in 20 liter tanks containing aquarium water (distilled water containing 1000 mg L^{-1} sea salt, 200 mg L^{-1} $CaCl_2$). Acclimation was performed in a temperature controlled room at 25 ± 1°C, with a 12-h light: 12 h dark cycle.

3.5 Sample Preparation and Instrumentation

3.5.1 Extraction and detection studies

3.5.1.1 Sample Preparation

For extraction and preconcentration experiments, ionic liquid, a novel solvent, was used. Details about the physical and chemical properties of ionic liquid are mentioned elsewhere in the thesis (Refer to Chapter 4). Ionic liquid (BMiM PF_6, 100 µL) was added

to nanopure samples spiked with MCLR (50 µg L^{-1}). The reaction mixture (5 mL) was adjusted to pH 3 with dilute HCl. It was kept in the water bath maintained at a specific temperature (65°C) for a stipulated amount of time (10 min). The tubes were taken out and cooled rapidly in water maintained at 4ºC. After cooling, the mixture became turbid and resulted in an emulsion. The tubes were later centrifuged at 4000 rpm for 5 min. A biphasic mixture was obtained. The ionic liquid-rich phase was separated and diluted with 100 µL of methanol and 10 µL was directly injected for the analysis of MCLR and MCRR using LC-MS-MS.

3.5.1.2 Instrumentation

MCLR and MCRR concentrations were determined by a liquid chromatograph (LC), composed of an HP100 liquid chromatograph (Agilent Technologies, U.S.A) interfaced with a triple quadrupole MS-MS (Applied Biosystems, U.S.A). Analytical separation was achieved on a Zorbax Extend-C18 5 µm, 2.1 x 150 mm (Agilent technologies, Germany). The injection volume was 10 µL. The mobile phase consisted of 0.1 % formic acid (solvent A) and methanol (solvent B). A gradient elution was used, starting with water: methanol at 90:10 from 0 to 6 min, and switching to 5:95 up to 10 min before returning to the original conditions to re-equilibrate the system. The capillary voltage was set at 89.00 volts and the cone voltage at 4.00 volts. The desolvation gas (nitrogen) temperature and flow-rate were set at 350°C and 6151/h, respectively. The ion source temperature was set at 120°C. LC-MS-MS was operated in the positive ion mode. MCLR and MCRR were monitored by using the MS instrument in the SRM mode (m/z 995.6 and fragment ion at 135.1; m/z 567.1 and fragment ion 135.1 respectively).

3.5.2 Biochemical studies

3.5.2.1 Sample preparation

After exposure, zebrafish were sacrificed and dissected. Gills, liver, intestine and brain from control and exposed animals were harvested, snap frozen in liquid nitrogen and stored at -80°C until measurements. Briefly, tissues were homogenized using 50 mM potassium phosphate buffer, pH 6.5 containing 1 mM EDTA. After removal of cell debris (14,000 g x 10 min, 4^{o}C), the resulting supernatant was used for the enzyme activity measurement.

Enzyme activities were determined by a colorimetric technique. The activity of GST (EC.2.5.1.18) was determined using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate (Sigma CS0410) (Habig et al., 1981). The GST assay kit utilizes CDNB which is suitable for the broadest range of GST isozymes. Upon conjugation of the thiol group of GSH to the CDNB substrate, there is an increase in the absorbance due to the formation of the reaction product, GS-DNB conjugate, at 340 nm which is measured. The rate of increase in the absorption is directly proportional to the GST activity in the sample.

$$GSH + CDNB \xrightarrow{GST} \text{GS-DNB Conjugate} + HCl \qquad (R1)$$

The enzyme activity was calculated using Eqn (1):

$$\text{Thioester formed (nmol min}^{-1}) = 5.3 \times DF \times \Delta OD\ min^{-1} \qquad \text{Eqn (1)}$$

To calculate ΔOD min^{-1}, absorbance was measured per minute at 340 nm. The following equation (Eqn 2) was used to calculate at ΔOD min^{-1} for samples.

DF: Dilution factor used for the reaction mix

OD: Optical density

$$\Delta OD\ min^{-1} = \frac{A340\ (final\ reading) - A340\ (initial\ reading)}{Reaction\ time\ (min.)} \quad (Eqn\ 2)$$

For CDNB emM : 5.3 $mM^{-1}cm^{-1}$

The activity of GR (EC 1.6.4.2) was measured by absorbance caused by the reduction of DTNB [5, 5-dithiobis (2-nitrobenzoic acid)] at 412 nm (Sigma GRSA) according to the procedure developed by Tanaka et al., 1994 (Tanaka et al., 1994). The underlying principle is based on the reduction of oxidized glutathione (GSSG) by NADPH (Nicotinamide adenine dinucleotide phosphate) in the presence of GR. In addition, DTNB reacts with the GSH formed:

$$NADPH + H+ + GSSG \xrightarrow{GR} NADP + 2\ GSH \quad (R2)$$

The reduced glutathione can then spontaneously react with DTNB:

$$GSH + DTNB \longrightarrow GS\text{-}TNB + TNB \quad (R3)$$

TNB = 5-thio (2-nitrobenzoic acid)

The second reaction was used for the assay by measuring the increase in absorbance at 412 nm, caused by reduction in DTNB using an extinction coefficient of 14.15 for TNB. The specific activity was expressed as nmol of NADPH oxidiz-ed/min/mg protein. The enzyme activity was calculated using the following Eqn (3):

$$NADPH\ oxidized\ (nmol\ min^{-1}) = (DF \times \Delta OD\ min^{-1}) / 14.15 \quad Eqn\ (3)$$

To calculate Δ OD min^{-1} , absorbance was measured per minute at 412 nm, and Eqn 4 was used to calculate Δ OD min^{-1} for samples

$$\Delta \text{ OD } min^{-1} = \frac{\text{A412(final reading)} - \text{A412 (initial reading)}}{\text{Reaction time (min)}} \qquad \text{Eqn (4)}$$

For TNB emM (extinction coefficient) = 14.15 $mM^{-1}cm^{-1}$

The activity of GPx (EC 1.11.1.9) was determined by an indirect method based on the oxidation of GSH to oxidized glutathione GSSG catalyzed by GPx, which is then coupled to the recycling of GSSG back to GSH utilizing GR and NADPH (β-Nicotinamide Adenine Dinucleotide Phosphate, Reduced). The decrease of NAPDH absorbance was measured at 340 nm according to the experimental procedure reported by Drotar et al. (1985).

$$\text{R-OOH} + 2\,\text{GSH} \xrightarrow{\text{GPx}} \text{R-OH} + \text{GSSG} + H_2O \qquad \text{(R4)}$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\,\text{GSH} + \text{NADP}^+ \qquad \text{(R5)}$$

Where R-OOH is organic peroxide.

To calculate the enzyme activity, the following Eqn (5) was used:

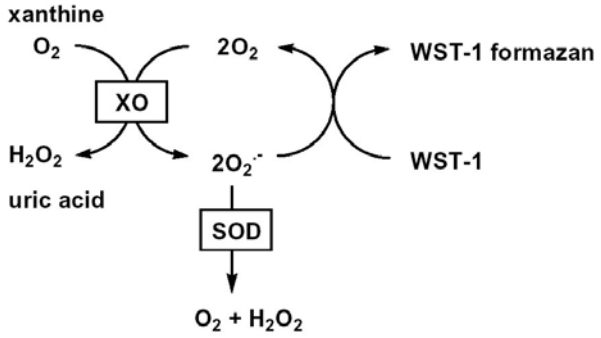
nmol of NADPH oxidized min^{-1} = ΔOD min^{-1} x DF / 6.22 Eqn (5)

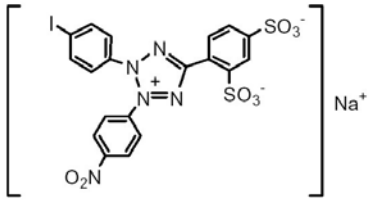
To calculate Δ OD min^{-1} , absorbance was measured per minute at 340 nm, and the following equation was used to arrive at Δ OD min^{-1} for samples (Eqn 6) :

$$\Delta \text{ OD min}^{-1} = \frac{\text{A340(final reading)} - \text{A340(initial reading)}}{\text{Reaction time (min.)}} \qquad \text{Eqn (6)}$$

For NADPH emM : 6.22 $mM^{-1}cm^{-1}$

SOD (EC 1.15.1.1), which catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen, was assayed by utilizing Dojindo's highly water soluble tetrazolium salt, WST-1(2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion.





The rate of reduction of WST with O_2 is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. Hence, the SOD activity was calculated as an inhibition activity quantified by measuring the decrease in the color development at 440 nm (Sigma, 19160) – Eqn (7):

SOD activity (inhibition rate %) = (A_{blank} - A_{sample}) / A_{blank}) x 100 Eqn (7)

A_{blank}: Absorbance of blank

A_{sample}: Absorbance of sample

The specific enzymatic activity (GST, GR and GPx) was calculated in terms of the protein content of the sample according to the method developed by Bradford (1976) (Bio-Rad 5000001), and is reported in nmol/ml/min/mg protein (Bradford 1976). This assay is a dye-binding assay in which a differential color change of a dye occurs in response to various concentrations of protein. The absorbance maximum for an acidic solution of Coomassie® Brilliant Blue G-250 dye shifts from 465 nm to 595 nm when binding to protein occurs. The intensity of absorbance measured at 595 nm is directly proportional to the amount of protein in the sample. The amount of protein in the sample was calculated using Beer Lambert's law using a standard calibration curve made with BSA (Bovine Serum Albumin).

3.5.2.2 Instrumentation

Enzymatic assays were carried out using a UV-Vis plate reader (bio-rad), equipped with temperature control and shaking. The assays were read in a kinetic mode, taking the reading from a 96-well plate every minute of the reaction.

3.5.3 Immunohistochemical studies

3.5.3.1 Sample Preparation

After exposure, zebrafish were slit open ventrally from the heart to the anus to expose the digestive organs. The entire fish was fixed in 10% formalin (HCHO) solution for 7 days.

The fish were kept in histology cassettes and then immersed in formalin solution. Solution was changed once every day. After fixation in formalin, they were washed in 70 % ethanol for 2 days. 70% ethanol was changed twice a day for the course of two days.

The washing step was followed by dehydration of samples in a graded series of ethanol before clearing in Histoclear and embedding in paraffin (Table 3.1).

Table 3.1:
Basic steps in processing of fixed fish samples for histology

Solvent Used/ Chemical	Duration (hours)
70% ethanol	1
90% ethanol	2
95% ethanol	1
100 % ethanol	1
100 % ethanol (repeat)	2
50% ethanol , 50% histoclear	1
Histoclear	1
Histoclear (repeat)	8
Paraffin	2
Paraffin (repeat)	8

After the paraffin step, the samples were embedded using a histokinetic machine in the histology cassettes and stored in 4° C until sectioning. The paraffin embedded tissues were then sectioned serially at 5-µm thickness (with the help of a microtome cutter) and used for immunohistochemical study.

For immunohistochemistry slides were immersed in staining jars with lids containing the appropriate chemical/reagent/stain for the entire experiment. The paraffin sections were first de-waxed in histoclear and then rehydrated in graded series of washes with ethanol and subsequently with water (Table 3.2).

Table 3.2
Basic steps in processing paraffin sections for immunohistochemistry

Solvent used /chemical	Duration (min)
De-wax in histoclear	10
De-wax in histoclear (repeat)	10
100% ethanol	2
100% ethanol (repeat)	3
90% ethanol	3
70% ethanol	3
50% ethanol	3
Milli-Q water	5
Milli-Q water (repeat)	5

After the de-waxing and rehydration of paraffin sections, epitope retrieval was done using sodium citrate buffer (10 mM sodium citrate in milli-Q water at pH 6) ; epitope retrieval is a pretreatment procedure often used prior to immunohistochemistry to improve staining by modifying the molecular conformation of 'target' proteins through an exposure of slide-mounted specimen material to a heated buffer solution). The buffer was heated. Once it reached the boiling temperature, slides with the sections were immersed into it. The buffer was again boiled for 3 min, and later changed to medium

heat treatment for another 2 min, finally to a low heat treatment for 20 min. Slides were then taken out and were allowed to cool at room temperature for about 30 min.

After epitope retrieval, sections on the slides received further treatments and washes with wash buffer (PBST) before loading the primary antibody (ADDA specific; AD4G2, monoclonal antibody) on the sections. Details are given in the flow chart below (Figure 3.5):

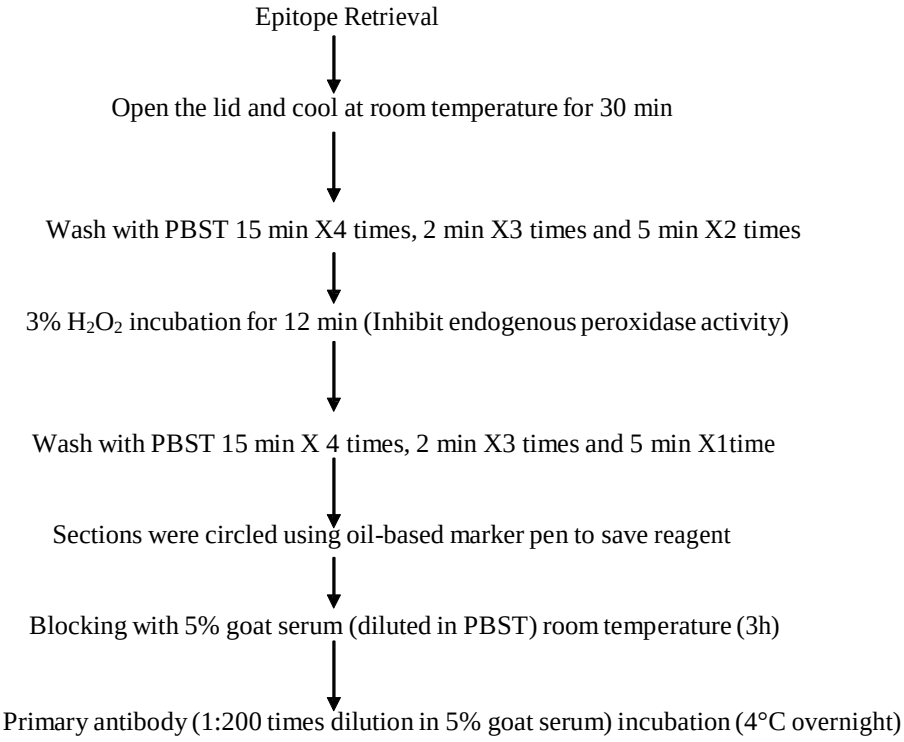


Figure 3.5: Steps for binding of primary antibody to section slides

PBST (20 X) was diluted 20 times with milli-Q to prepare its working solution. For blocking, goat serum was diluted (5%) with PBST and used as blocking agent. The primary antibody (ADDA specific; AD4G2, monoclonal antibody) was diluted to 200

times with goat serum before adding it to the section slides. The slides were then incubated overnight (4°C) in a humid slide box. After incubating with primary antibody overnight, the slides are washed thoroughly with subsequent steps for secondary antibody incubation and colour development (Figure 3.6)

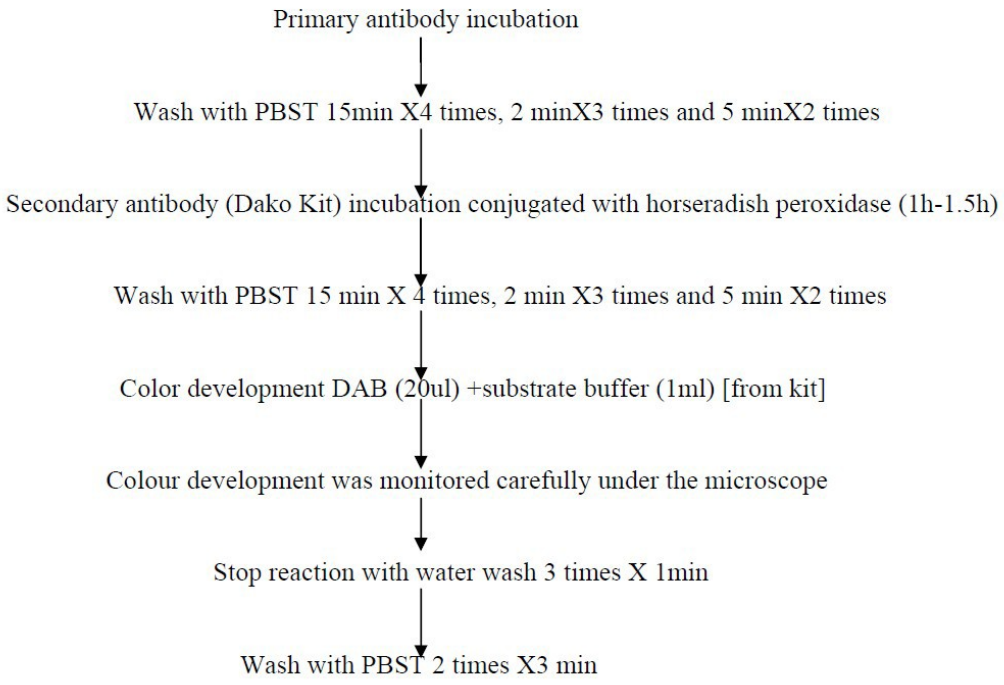


Figure 3.6: Steps for secondary antibody binding and colour development on sections

After primary antibody incubation, Rabbit anti-mouse antibody conjugated with horseradish peroxidase (Zymax grade, DAKO) for detection by 3,3- diaminobenzidine tetrahydrochloride (DAB) substrate-chromagen (DAKO) was used as a secondary antibody. Color development was carefully monitored under the microscope to avoid over staining. The reaction was stopped with waterwash as mentioned in Figure 3.6. After water and PBST wash, the section slides were counterstained with

hematoxylin (nuclear stain) for 1 min. After staining, the slides were washed under running tap water for 10 min and with PBST (2 min X 3 times). Subsequently, a series of dehydration steps using ethanol took place (50% - 70%-90% - 100% ethanol for 3 min each), followed by histoclear (5 min X 2 times). In the end, the slides were mounted using DPX mountant and covered with a coverslip. After mounting, slides were left to air-dry overnight. Once they were dried completely, they were viewed and captured under the microscope.

3.5.3.2 Microscopy

Images of the stained liver, gills and intestine sections (obtained from immunohistochemical analysis) were captured using Axiovert microscope (Zeiss) equipped with an imaging system.

3.5.4 Metabolomic studies

3.5.4.1 Sample preparation

For metabolomics, tissues were harvested after exposure and were freeze-dried using a lyophilizer (Applied biosytems, USA). After lyophilization, sequential extraction with solvents was done to extract the polar and non polar metabolites. A general workflow is given in Figure 3.7.

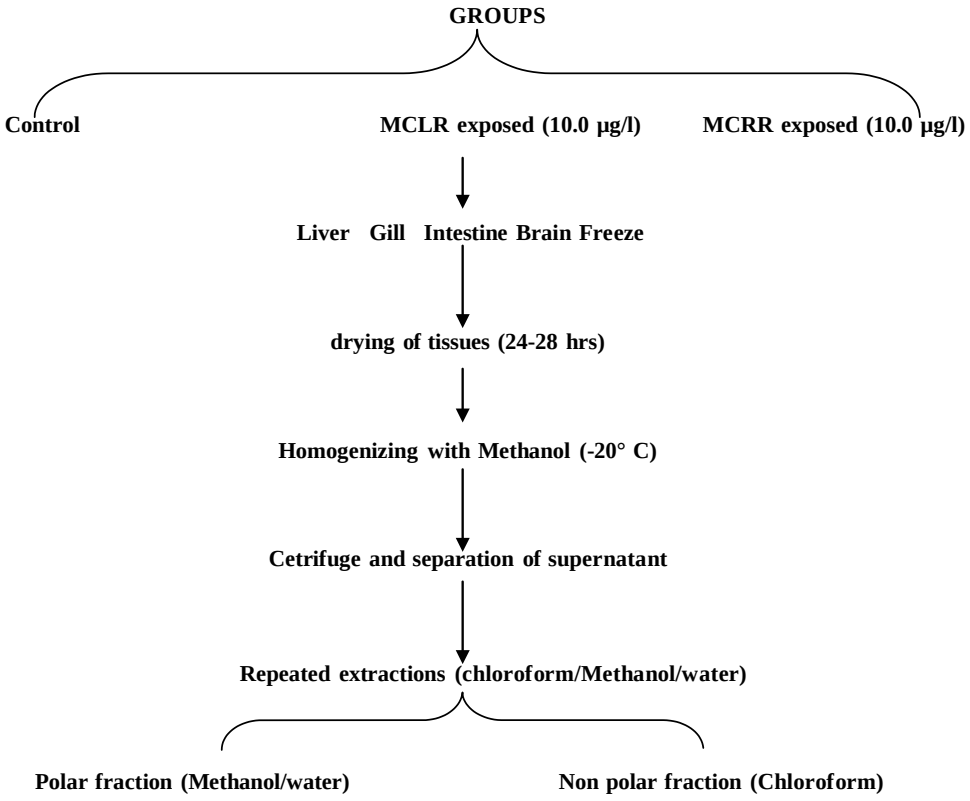


Figure 3.7: A general workflow for extraction of metabolites from zebrafish tissues

Following lyophilisation, tissue samples were homogenized with 250 µL of cold methanol (HPLC grade), maintained at -20°C. Samples were then homogenized on ice. 5 µL of two internal standard mixes were added to the samples at this stage. Internal standard mix 1 (consisted of DL-2-aminobutyric acid, 1-naphthylamine, 2-aminoanthracene, 2,3,4,5,6 -pentafluorobenzoic acid [0.01 mg mL^{-1} in 50% methanol]) was used for polar metabolites fraction while internal standard mix 2 (was composed of 9 deuterated lipid standards (16:0 D31-18:1 PE; 16:0 D31 Ceramide; Cholesterol (d7); 16:0 D31-18:1 PI; 16:0 D31-18:1 PG; 16:0 D31 -18:1 PS; 16:0 D31 -18:1 PC; 16:0 D31

SM; Sphingosine (d7)) used for non polar metabolites. Internal standard mix 2 was provided by Metabolomics Australia team. They were shipped on dry ice.

After addition of internal standards to the homogenised samples, samples were vortexed for 1 min and centrifuged at 14,000 rpm for 10 min at 4°C. After centrifugation, the clear supernatant was removed and transferred to another reaction tube on ice. The pellet from the first step was re-extracted again with another 250 µL of cold methanol. The pellets with methanol were then vortexed for 1 min and centrifuged (14,000 rpm; 10 mins at 4°C). Supernatants from first and second extraction were pooled together. 500 µL of milli-Q water was added to the pooled methanol supernatants. After that, 400 µL of chloroform was added to the sample which resulted in a biphasic mixture which was then vortexed briefly and centrifuged (14,000 rpm; 10 min at room temperature). After centrifugation, the reaction mixture was divided into two clear layers with an interphase composed of proteins and other debris. Upper polar phase consisting of methanol and water was carefully removed into another fresh reaction tube. The interphase was removed, and the non-polar phase supernatant was retained in the same tube. To that polar phase supernatant, another 300 µL of chloroform was added, vortexed briefly and centrifuged (14,000 rpm; 10 min at room temperature). After centrifugation, the clean upper polar phase was transferred to a fresh reaction tube. The residual interphase consisting of proteins and amino acids was removed and the non polar phase (chloroform) supernatant was pooled with the previous chloroform phase. After solvent-water extraction, the individual extracts (polar and non polar) were dried down using speedvac (Thermo scientific, USA).

The dried polar extracts were reconstituted using 50 µL of 50% methanol and were briefly vortexed. The samples were then sonicated in a room temperature water-bath for 3 min before being centrifuged at 13,200 rpm for 3 min at ambient temperature. The resulting supernatant was then transferred to glass vial inserts in LC-MS vials for instrumental analysis.

The dried non polar extracts were reconstituted using 100 µL of 50:50 methanol: butanol containing 10 mM ammonium formate and were briefly vortexed. The samples were then incubated in a shaking heater block for 25 mins at 30^{o}C before being centrifuged at 13,200 rpm (revolutions per minute) for 3 min at ambient temperature. The resulting supernatant was transferred to glass vial inserts in amber LC-MS vials for instrumental analysis.

3.5.4.2 Instrumentation

Polar extracts were analysed by RP-LC-MS (Reverse phase- Liquid chromatography-mass spectrometry) in positive mode and RP-LC-MS in negative mode in an untargeted or "profiling" manner using the instrumentation and methods as described here. Polar extracts were run through a HPLC (High Perfomance Liquid Chromatography; Agilent 1200 series with binary pump, U.S.A., equipped with well-plate autosampler and Q-TOF (q-Time of flight) Mass Spectrometer (Agilent 6520 Q-TOF LC/MS, U.S.A.). Separation of metabolites was achieved on a ZORBAX Eclipse Plus C18, Rapid Resolution HD 2.1 x 100mm, 1.8-micron (Agilent technologies, Germany). The injection volume was 5 µL. The mobile phase consisted of 0.1 % formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). A gradient elution was used, starting with A: B at 95:5

from 0 to 9 min, and switching to 0:100 at 10 min and holding for 2 min before returning to the original conditions to re-equilibrate the system. The capillary voltage was set at 4000 volts. The drying gas (nitrogen) temperature and flow-rate were set at 300°C and 10 L min^{-1}, respectively. The nebulizer pressure was set at 45 psi. After the sample injection, the data was mined, analyzed and interpreted using various softwares as discussed in section 3.8.

Non-polar extracts were analyzed by lipidomics methods in positive mode and in negative mode in an untargeted or "profiling" manner using the instrumentation and methods described here. HPLC (Agilent 1200 series; Binary pump with thermostated autosampler) equipped with Q-TOF Mass Spectrometer (Agilent 6520 Q-TOF LC/MS) was used in positive and negative modes for the non polar metabolite analyses. Separation of metabolites was achieved on a Ascentis Express RP Amide, 2.1 x 50 mm, 2.7 micron (Supelco, Sigma Aldrich, U.S.A.). The injection volume was 5 µL. The mobile phase consisted of solvent A (Water: Methanol: Tetrahydrofuran : : 50:20:30 in 10 mM ammonium formate) and solvent B (Water: Methanol: Tetrahydrofuran : : 5:20:75 in 10 mM ammonium formate). A gradient elution was used, starting with A: B at 100:0 at 0 min and switching to 0:100 at 8 min and holding for 2.2 min before returning to the original conditions to re- re-equilibrate the system. The capillary voltage was set at 4000 volts. The drying gas (nitrogen) temperature and flow-rate were set at 325°C and 10 L min^{-1}, respectively. The nebuliser pressure was set at 45 psi.

3.5.5 Gene expression studies

3.5.5.1 Sample Preparation

For gene expression studies, after the exposure, RNA was extracted from the tissue samples and further processed for microarray experiments. A general workflow adopted for Gene expression studies is given in Figure 3.8.

3.5.5.1.1 RNA (Ribonucleic acid) extraction

Tissue samples were first homogenized with a hand held motorized homogenizer on ice with 1 mL of trizol® reagent. The amount of RNA extracted from an individual tissue was not sufficient for the experiment, and hence, two tissues were pooled to obtain one biological replicate. Following homogenization, isolation step was done to remove proteins, fat, polysaccharides and other debris from the tissue sample. The insoluble fraction from the homogenate was removed by centrifugation at 12,000 $\times$ *g* for 10 minutes at 4°C. The resulting pellet contained extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contains RNA. In each case, the cleared homogenate solution was transferred to a fresh tube. After transferring, the homogenates were incubated at room temperature for 5 min, following which; 0.2 mL of chloroform was added to the tubes with vigorous shaking for 15 seconds. The samples containing chloroform were then incubated for another 3 min and centrifuged at 12,000 $\times$ *g* for 10 min at 4°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube; RNA was precipitated from the aqueous phase by adding 0.5 mL isopropyl alcohol.

Samples were then centrifuged at 12,000 × *g* for 10 min at 4°C. The RNA precipitate, often invisible before centrifugation, forms a gel-like pellet on the sides of the tube. The supernatant was removed. The RNA pellet was washed once with 75% ethanol, and was mixed by vortexing. It was then centrifuged at 7500 *X g* for 5 min at 4°C. After centrifugation, ethanol was removed and was air dried. The pellet was then dissolved in RNA-ase free water (20 µL) and incubated for 4 min at 55°C. Later it was checked for quality and quantity using nanodrop and bioanalyzer (Agilent bioanalyzer) as discussed below:

3.5.5.1.2 RNA quantity, quality and integrity

Quality, integrity and quantity of the isolated RNA were also estimated by using the RNA/DNA/Protein-analyser (Agilent BioAnalyzer 2100). The Agilent BioAnalyzer 2100 allows electrophoretic separation and fluorescence detection of labelled RNA on microfabricated chips. Rapidity and automation are some of the advantages of this method over a classic gel-electrophoresis. Specially devised software algorithm extracts information about total RNA sample integrity from entire electrophoretic lane and converts the information into a RIN value (RIN - RNA integrity number) on a scale from 1 to 10 (from completely degraded to highly intact RNA). Only intact RNA samples with RIN ≥ 8 were taken for further applications. For the RNA analysis in the BioAnalyzer, the RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, USA) was filled with a mixture of gel matrix and a fluorescent dye (both supplied in Agilent RNA 6000 Nano Reagents Kit), and consequently 1 µl of each RNA sample was added to a corresponding

well of the chip. One well of the chip was loaded with 1 µl RNA-ladder (RNA 6000 Nano Ladder, Agilent Technologies, Palo Alto, USA) which was used as a size standard.

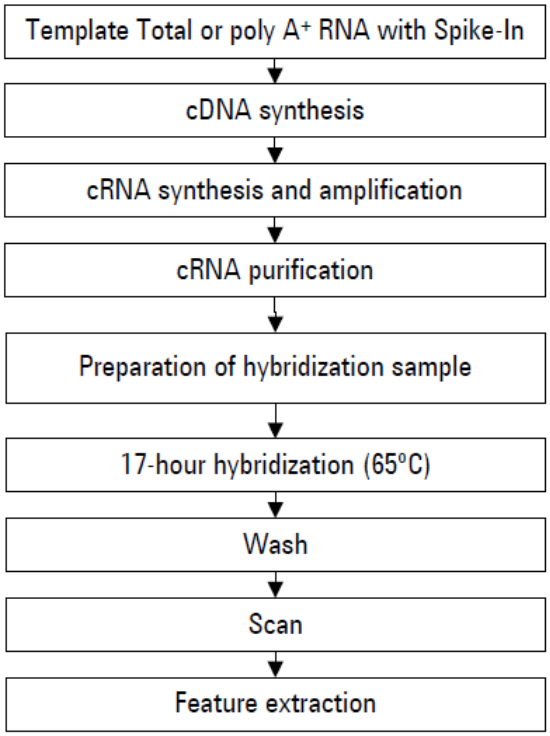


Figure 3.8: General workflow for microarray experiments (Taken from Agilent One color microarray based gene expression analysis manual)

3.5.5.1.3 Fluorescent cDNA (complimentary deoxyribonucleic acid) synthesis

The RNA concentration of tissue samples used for cDNA synthesis was 400 ng. RNA from tissue samples were diluted using RNA-ase free water and their concentrations were adjusted to 400 ng. Care was taken that the final sample taken for synthesizing cRNA was no more than 6 µL after adjusting with water. From Agilent One-Color RNA spike-in Kit, the Agilent One-Color Spike-mix tube was vigorously mixed and vortexed. Then it was serially diluted (according to the manual instructions) using a dilution buffer

provided in the kit. From the third dilution, 4 µL of the spike- in mix was added per reaction/sample. 1.2 µL of T7 promoter primer was then added per reaction. After adding the spike-in mix and T7 promoter primer to each reaction, the samples were denatured by incubating then at 65°C for 10 min. Reactions were then placed on ice for 5 min following the addition of cDNA master mix (4 µL of 5 X strand buffer; 2 µL of 0.1 M DTT (Dithiothreitol);1 uL of 10 mM dNTP (deoxyribonucleotide triphosphate); 1 µL of MMLV-RT (Moloney Murine Leukemia Virus Reverse Transcriptase); 0.5 µL of RNA-ase out per reaction). Total cDNA master mix added per reaction was 8.5 µL (rewrite it). After the addition, samples were incubated at 40°C and 65°C for 2 h and 15 min, respectively in a circulating water bath. Samples were then taken out and incubated on ice for 5 min.

3.5.5.1.4 cRNA synthesis and amplification

After incubation, 60 µL of transcription master mix was added to the samples (Mix consisted of 15.3 µL of nuclease-free water; 20 µL of 4 X transcription buffer; 6 µL of 0.1 M DTT; 8 µL of NTP mix; 6.4 µL of 50% PEG (Polyethyleneglycol); 0.5 µL RNase out; 0.6 µL of inorganic pyrophosphatase; 0.8 µL of T7 RNA polymerase ; 2.4 µL of Cyanine 3-CTP dye). Following the addition, samples were incubated at 40°C for 2 h.

3.5.5.1.5 Purification of labeled/amplified RNA

After incubation, the cRNA samples were purified. Qiagen's RNeasy kits (mini spin columns) were used for purification of the amplified cRNA samples. 20 µL of nuclease-free water was added to cRNA sample, following which, 350 µL of Buffer RLT was added and mixed well by pipetting. Later, 250 µL of 100 % ethanol was added to the

cRNA samples containing nuclease-free water and buffer RLT. 700 µL of the cRNA sample was transferred to an RNeasy mini column in a 2 mL collection tube and centrifuged at 4°C for 30 seconds at 13,000 rpm. Flow through coming from the mini spin columns got accumulated in the collection tube. This flow through in the collection tube was discarded and the purification cycle was repeated after adding 500µL RPE buffer to the sample (twice). RNeasy column was then transferred to a new 1.5 mL collection tube and sample was centrifuged again at 4°C for 30 seconds at 13,000 rpm to remove any remaining traces of buffer RPE. Then the collection tube was discarded and fresh tube was used to elute the cleaned cRNA sample. Elution of the cleaned cRNA sample was done by transferring the RNeasy column to a new 1.5 mL collection tube. 30 µL of RNase-free water was then added directly onto the RNeasy filter membrane. After 60 sec, the collection tubes were centrifuged at 4°C for 30 seconds at 13,000 rpm. Purified cRNA samples thus obtained in flow-through were maintained on ice.

3.5.5.1.6 Quantification of cRNA

cRNA was quantified using nanodrop (ND-1000-UV-VIS) spectrophotometer. It was quantified under 'microarray measurement' tab. RNA-40 was selected as the sample type. Cyanine- 3 dye concentration (pmol/µL), RNA absorbance ratio (260nm/280 nm) and cRNA concentration (ng/µL) was determined by the instrument as given in Eqn (8)

Concentration of cRNA was used to determine the yield of cRNA (µg cRNA) as follows:

(*Concentration of cRNA*) * 30 µL (*elution volume*) / 1000 = µg of cRNA Eqn (8)

Concentrations of cRNA (ng/µL) and cyanine 3 (pmol/µL) were used to determine the specific activity as follows (Eqn (9)):

(*Concentration of Cy3*) / (*Concentration of cRNA*) * 1000 = pmol Cy3 per µg cRNA if the yield was <1.65 µg and the specific activity was < 9.0 pmol Cy3 per µg of cRNA, then entire process of cRNA preparation was repeated.

3.5.5.1.7Hybridization

If the yield and specific activity were as per the requirement (as mentioned above)-unclear, the samples were then used for hybridization. For hybridization, the fragmentation reaction mix for each sample was formed with the following constituents: 1.65 µg of cRNA obtained from earlier step, 11 µL of blocking reagent, nuclease-free water (to bring the volume to 52.8 µL) and 2.2 µL of 25 X fragmentation buffer (all the regents were provided in the Agilent gene expression hybridization kit). The total volume of fragmentation reaction mix was 55 µL. The tubes containing the reaction mix were incubated for exactly 30 min at 60°C. Following the incubation, the reaction was immediately stopped by the addition of hybridization buffer (55 µL). It was mixed well with pipetting and spun for 1 min at 13,000 rpm (room temperature). Tubes containing the reaction mix were then placed on the ice and loaded on the microarrays.

3.5.5.1.8 Hybridization assembly and chamber

After loading the samples on the microarrays, they were hybridised in the hybridization chamber by Agilent technologies (U.S.A.) with the oven set at 65°C, rotating at 10 rpm

for 17 hours. After hybridisation, the microarrays were washed with Gene expression wash buffers (provided by Agilent, following the manufacturer's instructions).

3.5.5.2 Instrumentation

After the washing, the microarrays were scanned and features were extracted as discussed below:

3.5.5.2.1 Scanning the microarray slides

Scanning and feature extraction of the hybridized and washed microarray slides were done on Agilent Microarray scanner (GenePix 4000 B; Agilent technologies, U.S.A.). The slides were assembled into an appropriate slide holder and scan settings for one color scans were verified, they were set as follows: Scan region – 61 X 21.6 mm; scan resolution- 5 µm; scanning mode – single pass; dye channel – green ; greet PMT : XDR hi 100%, XDR lo 10%.

3.5.5.2.2 Feature extraction

After scanning the microarray slides, feature extraction was done using Agilent feature extraction software which was installed on the computer synced with the Agilent scanner. Feature Extraction is the process by which information from probe features is extracted from microarray scan data which allows to measure gene expression in the experiments. After generating the microarray scan images, features were extracted using Feature Extraction protocols for gene expression provided on Agilent Web site at www.agilent.com/chem/feprotocols. After feature extraction, the data was normalized, analyzed and interpreted using Genespring software provided by Agilent technologies, U.S.A (Section 3.7).

3.6 Kits

3.6.1 Biochemical studies

Four enzyme kits, namely, glutathione peroxidise (GPx), glutathione reducatase (GR), superoxide dismutase (SOD) and glutathione-S-transferase (GST) were procured from Sigma Aldrich Corporation (USA) for biochemical studies on fish tissue samples exposed to MCLR/MCRR. Protein assay kit was purchased from Bio-Rad (U.S.A.). Details about the usage and working principle are given elsewhere in Section 3.5.2.

3.6.2 Immunohistochemical studies

Rabbit anti-mouse antibody (secondary antibody) conjugated with horseradish peroxidise kit, zymax grade for immunohistochemistry was obtained from DAKO (Denmark). Details about the usage and working principle are given elsewhere in Section 3.5.3.

3.6.3 Gene expression studies

Quick Amp Labeling Kit (One-Color) ,RNA Spike-In Kit (One-Color), Gene Expression Hybridization Kit and microarray slides for microarray experiments were purchased from Agilent Technologies, U.S.A. RNeasy mini kits for the experiments were purchased from Qiagen, Netherlends. Agilent RNA 6000 Nano Reagents Kit for evaluating the quality and integrity of RNA samples was purchased from Agilent Technologies; U.S.A. Details about the usage and working principle are given elsewhere in section 3.5.4.

3.7 Specialized consumables/ accessories

The following consumables were purchased: centrifuge tubes (15 and 50 ml) from B.D. Falcon (U.S.A); Reaction tubes (1.5 and 2 ml), pipette, pipette tips and pipette tip boxes from Eppendorf (Germany); Cryovials and cryo storage boxes Nunc (Denmark) ; Injection needles from Braun (Germany); Microscopic slides from Superfrost (Germany); 96-well plates (Nunc, Germany); Petridishes from Grener (Germany); GC vials (2 ml) and vial inserts from Agilent technologies (U.S.A.); Parafilm from Pichiney (U.S.A.); Histology cassettes, histology molds, write-on markers, slide storage boxes from Simport (Canada); Super PAP liquid blocker pen for histological slides from Daido Sangyo Co. Ltd., (Japan). High profile disposable blades for sectioning were purchased from Leica Microsystems (Germany). A hand held motorized homogenizer with disposable pestles was purchased from Kontes, U.S.A. Dissection tools and accessories were obtained from Ted Pella Inc, U.S.A.

3.8 Softwares Used

3.8.1 Analyst

Analyst was used for peak detection and quantification of MCLR and MCRR in samples (for method development; detection of MCs)

3.8.2. Graph pad prism

Graph pad prism was used for statistical analyses of data obtained from biochemical set of studies done on zebrafish tissues under balneation conditions (exposure to MCLR and MCRR)

3.8.3. MZ mine

This software was used to mine the data from the analytical instrument for polar and non polar metabolite (metabolite studies) extracted from organs of zebrafish. Details about the software are given in Chapter 7.

3.8.4 Software 'R' for data visualization and interpretation (Metabolomic studies)

The software, R, was used for normalization, visualization and interpretation of data obtained from metabolite studies. R is a freeware available on the internet. The data can be visualized and statistically analyzed using 'R scripts'. Details about the software and its usage in this study are given in Chapter 7.

3.8.5 Genesrping

Genespring was used for mining, normalization, visualization, statistical analyses of data obtained from microarray experiments (Gene expression studies). Details about the software are given in Chapter 8.

CHAPTER 4

DEVELOPMENT OF A SENSITIVE ANALYTICAL METHOD FOR SIMULTANEOUS DETECTION OF MCLR AND MCRR

This chapter describes a development of an analytical method for extraction and detection of MCLR and MCRR from natural waters. This method involves a novel solvent, ionic liquid, which contains a cation and an anion moiety. This ionic liquid was employed to extract and preconcentrate MCLR and MCRR under optimum conditions. The conditions for achieving maximum extraction efficiency were optimized in these experiments for highest sensitivity. After optimization, the developed analytical method was validated with spiked real water samples.

4.1 Introduction

Before evaluating the toxicological implications of extracellular MCLR and MCRR, it is important to investigate and evaluate the relative concentrations of these MCs in local reservoirs**.** In addition, from health risk assessment and management perspective, there is a need for developing a selective, sensitive, simple and reliable analytical method that is capable of measuring both MCLR and MCRR under local environmental conditions.

Various biochemical (Nagata et al., 1997, Pyo et al., 2005, Metcalf et al., 2001) and physiochemical methods (Lawton et al., 1994, Meriluoto et al., 1998, Bateman et al., 1995, Sano et al., 1992) have been used over the years to detect intracellular and extracellular MCLR and MCRR in natural waters. WHO safety guideline for extracellular MCLR is set at 1 µg L^{-1}. However, direct detection of such low levels of MCLR is not

feasible on a routine basis due to inadequacy of previously developed detection methods which tend to have a lag between the sampling and delivery of the results and hence, such methods are cumbersome (Lawton et al., 1994a; Lawton et al., 2010). In most of the physiochemical methods developed earlier, preconcentration of water samples is required prior to chemical analysis. Solid phase extraction (SPE) and size exclusion chromatography (SEC) are typically used to enrich environmental concentrations of MCs, or to eliminate other contaminants from complex samples such as animal and plant tissues followed by HPLC/UV or LC/MS (+ESI) detection modes (Hummert et al., 1999; Cong et al., 2006; Barco et al., 2002; Xu et al., 2008; Man et al., 2002). Details about extraction and preconcentration of MCs are given elsewhere in chapter 2 (Literature Review). However, these preconcentration methods are relatively cumbersome and time consuming as mentioned before. Moreover, some of these methods utilize toxic, hazardous and volatile organic solvents, which are not environmentally friendly. Alternative environmentally friendly preconcentration methods are critically needed without compromising the sensitivity and specificity of the conventional methods. Moreover, development of reliable, rapid and sensitive methods for the identification and quantification of MCs in the aquatic environment would help in routine water quality monitoring and thus in improving the quality of natural waters when HABs occur.

In the present study, attempts were made to explore the possibility of performing simultaneous extraction and preconcentration of MCLR and MCRR using green solvents. Room temperature ionic liquids (RILs) are gaining recognition as unique solvents for environmental applications due to their favorable properties (Marsh et al., 2004). RILs result from the combination of organic cations and anions that may be liquids at room

temperature. These RILs have melting points below ca. 100 ºC (Pandey, 2006; Seddon et al., 2000). RILs have excellent physiochemical properties such as negligible vapor pressure, good thermal stability, tunable viscosity, negligible volatility and miscibility with water and organic solvents which make them suitable for application in environmental analytical chemistry (Poole 2004). The main advantage of ionic liquids is that they are a new class of solvents by their non-molecular nature. The ionic liquid environment is very different of that of all molecular polar or non-polar organic solvents (Fitzwater et al., 2005). The intrinsic non-molecular nature of RILs gives them unique solvent properties, making then suitable to be used at places where separation, extraction and concentration of analytes are required (Visser et al., 2001; Liu et al., 2003). Moreover, extremely low volatility of RILs renders them little flammable so they could be an ideal candidate to replace organic pollutant solvents in all the above mentioned processes (Chauvin and Olivier-Bourbigou 1995). As mentioned earlier, RILs are combination of anions and cations. Most commonly considered cations for extraction in RILs are those based on the imidazolium ($C_3H_4N_2$) or pyridinium (C_5H_5N) ring with one or more alkyl groups attached to the nitrogen or carbon atoms. However, quaternary ammonium salts have also been widely used for electrochemical synthesis. Most commonly considered anions for extraction in RILs include halide ions, tetrafluoroborate (BF_4^-), tetrachloroaluminate($AlCl_4$), hexafluorophosphate (PF_6^-), and bis(perfluoromethyl-sulfonyl)imide anion $(CF3SO_2)_2N^-$ (also known as bistriflate imide (Tf_2N^-)). In this study, imidazolium cation and hexafluorophosphate anion (BMiM PF_6) was used as a model representing ionic liquid family.

4.2 Methodology

4.2.1 Extraction Procedure

Chemicals, reagents and instrumental conditions used in these set of experiments are given in Chapter 3 (Section: 3.2.1, 3.2.2 and 3.5.1). Briefly, 100 µL of ionic liquid was added to 5 mL of nanopure samples (pH 3) spiked with MCLR (50 µg L^{-1}). It was then heated at 65°C for 10 min. The tubes were cooled and were later centrifuged at 4000 rpm for 5 min. The ionic liquid-rich phase was then separated and diluted with 100 µL of methanol and 10 µL was directly injected for the analysis of MCLR and MCRR using LC-MS-MS. Extraction procedure details are also given in Chapter 3, section 3.5.1. Different parameters affecting the extraction procedure were optimized using MCLR as a reference toxin. For MCRR, optimized conditions were used for recovery tests and validations. Since MCLR and MCRR are structurally very similar, MCLR was used for optimization experiments.

4.2.2 Validation using real water samples

Reservoir samples were collected from different sites in Singapore and were spiked with known concentrations of MCLR and MCRR (10 µg L^{-1}) and were allowed to age for 24 hours at 4°C before the extraction. Recoveries were determined for the developed method using the optimized procedure. Moreover, water samples were also evaluated for the presence of MCLR and MCRR. Physiochemical properties were also examined in all the water samples.

4.3 Results and discussion

The factors that affect the cloud point extraction such as extraction temperature, extraction time, the amount of ionic liquid and amount of extraction volume and sample pH were investigated using spiked nanopure water samples_ Each sample was analyzed six times under identical experimental conditions to confirm the reproducibility of analytical data. The results were reported with standard deviations (Figures 4.1 to 4.5). The extraction efficiency was calculated based on the area of the LC-MS-MS peaks; larger peak areas correspond to higher efficiency. To evaluate the practical applicability of the method, linearity, limits of detection (LOD), recoveries and relative standard deviations were measured under the optimized extraction conditions. Blank extractions were conducted at regular intervals of method development to assess the contamination of the glassware and carryover effect.

4.3.1 Effect of temperature and time for extraction

MCs are found to be stable at very high temperatures (Harada et al., 1996). Yu et al. (2009) found that after incubating MCLR for 6 hours, it was still stable when the temperature was less than 100 °C (Harada et al., 1996; Yu et al., 2009). In view of this finding, the effect of various temperatures was studied on the extraction efficiency as extraction temperature is a key for cloud point extraction. The method optimization was therefore initiated by studying the behavior of the ionic liquid with MCLR in water at different time and temperature intervals. Briefly, the tubes were kept at different temperatures ranging from 25 to 75°C for 10 min maintained in a water bath. It was found that the peak area increased with the increase in temperature, reaching the

maximum at 65 °C and then decreasing markedly (Figure 4.1). Above 45°C, it was observed that after incubation for 10 min, the reaction mixture became homogenous, and the coexistence of two separate isotropic phases was not observed. After incubation, when the tubes were cooled in water at 4°C, the solution became turbid in 2-3 min, resulting in an emulsion, which was made up of a continuous phase and a dispersed phase. In the present case, water serves as a continuous phase while the ionic liquid serves as a dispersed phase. The interface could be the region for interaction between the MCLR and the ionic liquid which resulted in greater extraction efficiency for a temperature range where turbidity was observed on cooling. For further experiments, 65°C was therefore used as the temperature for extraction.

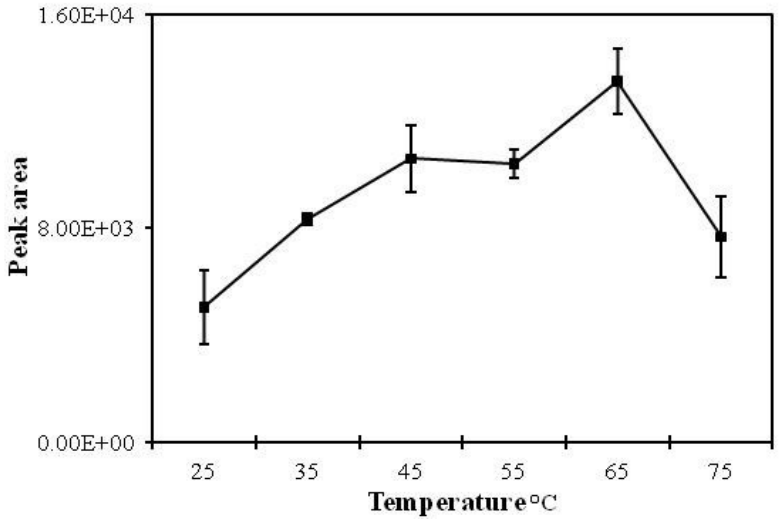


Figure 4.1: Temperature effect on extraction efficiency (5 mL reaction mixture containing spiked MCLR (50 µg L^{-1}) with 100 µL of BMiM $PF_{6;}$ incubated for 10 min at different temperatures. Ionic liquid phase injected in LC/MS/MS water (0.1% formic acid): methanol: 90:10; 0.3 mL min^{-1}).

The effect of heating time was also studied to determine the maximum extraction efficiency. It was observed that beyond 10 min there was a decrease in the peak area for the entire temperature range studied (Figure 4.2). It was therefore decided to carry out the extraction of MCLR at 65°C for 10 min as these conditions provided the highest peak area and the maximum extraction efficiency.

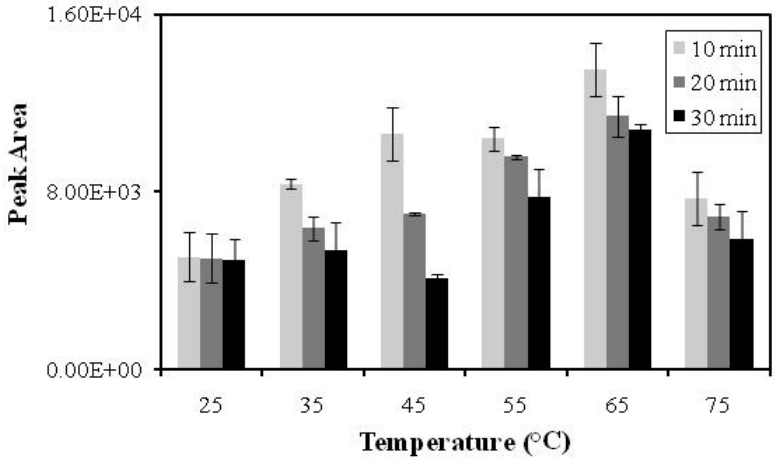


Figure 4.2 : Effect of varying time on extraction efficiency (5 mL reaction mixture containing spiked MCLR (50 µg L^{-1}) with 100 µl of BMiM PF_6 maintained at pH 3; incubated for 10, 20 and 30 min at different temperature . Ionic liquid phase injected in LC-MS-MS water (0.1% formic acid): methanol: 90:10; 0.3 mL min^{-1}).

4.3.2 Effect of pH on extraction

Since the structure of MCLR and MCRR contains numerous ionizable groups, the overall charge on the toxin is dependent on the pH of the medium. De Maagd et al. (1999) demonstrated that the MCLR species remain neutral at the narrow pH range of 2.09 - 2.19; the cationic species [$(COOH)_2(NH_2^+)$] are produced at pH < 2.09 and anionic species, [$(COO^-)_2$ (NH_2^+)] and [$(COO^-)_2$ (NH)] are produced at pH > 2.19 (De Maagd et al., 1999). Better extraction efficiencies are expected at pH values ranging from 1 - 2.09 to 3 - 12 considering the structure of ionic liquid which has active cationic and anionic

species. These species could possibly undergo ion pairing with charged head groups of MCLR. However, it was observed that the peak area increased from pH 1 to 3, with the maximum at 3, followed by a decrease with further increase in pH (Figure 4.3). This trend could be explained in terms of partitioning of the charged MCLR between ionic liquid and water at different pH values. The degree of partitioning is an indicator of differential solubility of MCLR between two immiscible solvents at equilibrium. Normally, one of the solvents chosen is water and the other one is hydrophobic, for example, octanol. The partitioning behavior between solvents correlates with the tendency of a molecule to concentrate in the lipids of organisms and the organic carbon of sediments and soils (Carda-Borch et al., 2003). De Maagd et al. (1999) demonstrated that the n-octanol/water distribution ratio, $D_{ow,}$ for MCLR decreased from 2.18 at pH = 1 to -1.76 at pH = 10. This ratio suggests that with the increase in pH, MCLR exhibits increased hydrophilicity and consequent decreased partitioning in octanol. Carda-Borch et al. (2003) investigated the ionic liquid (BMiM PF_6)/water distribution coefficients (IL/W) and compared them with the corresponding octanol/water distribution coefficients (O/W) for a pH range. They found that the IL/W distribution coefficients are generally higher for compounds containing amine groups than the O/W distribution coefficients for a pH range of 2-10 (Carda-Borch et al., 2003). This could be the possible explanation for highest extraction efficiency observed at pH 3 in the present case while the D_{ow} observed for MCLR was highest at pH 1. pH 3 was therefore used for the reaction mixture for further experiments. There are no reports detailing the behavior of MCRR at various pH. However, since MCLR and MCRR differ only in one amino acid, the behavior pattern

should be similar as for MCLR. With this in mind, the same optimized pH was used for MCRR extraction as well.

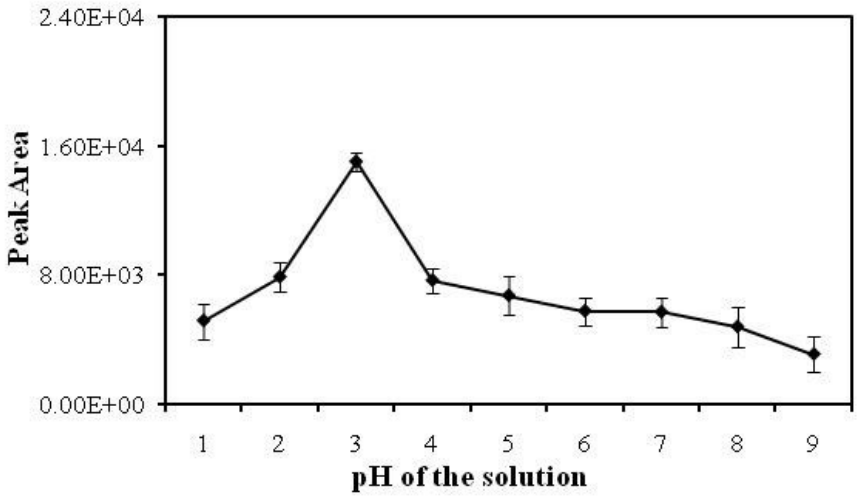


Figure 4.3: Effect of pH on the extraction efficiency (5 mL reaction mixture containing spiked MCLR (50 µg L^{-1}) with 100 µl of BMiM PF_6 at different pH; incubated for 10 min at 65ºC. Ionic liquid phase injected in LC/MS/MS water (0.1% formic acid): methanol: 90:10; 0.3 mL min^{-1}).

4.3.3 Effect of concentration of ionic liquid

This is one of the important parameters to be considered while developing an analytical method which involves pre-concentration and simultaneous extraction. Different volumes of the ionic liquid (75 to 250 µL) were used, keeping the other extraction parameters unchanged (Figure 4.4). Less than 75 µL of the ionic liquid could not be used as it gets dissolved in the reaction mixture at 65°C and does not form the distinct phase upon cooling. This observation could be explained in terms of the solubility of BMiM PF_6 in water which is higher than that of the other ionic liquids such as 1-Hexyl-3-methylimidazolium hexafluorophosphate (HMIM PF_6) and 1-Octyl-3- methylimidazolium hexafluorophosphate (OMIM PF_6) (Chun and Dzyuba 2001). It was observed that

the extraction efficiency increased from 75 µL to 100 µL, remaining high at 150 µL and then decreasing at 200 µL. The decrease could be attributed to increased dilution and the corresponding decrease in the pre-concentration factor. For further, experiments, 100 µL of the ionic liquid was used.

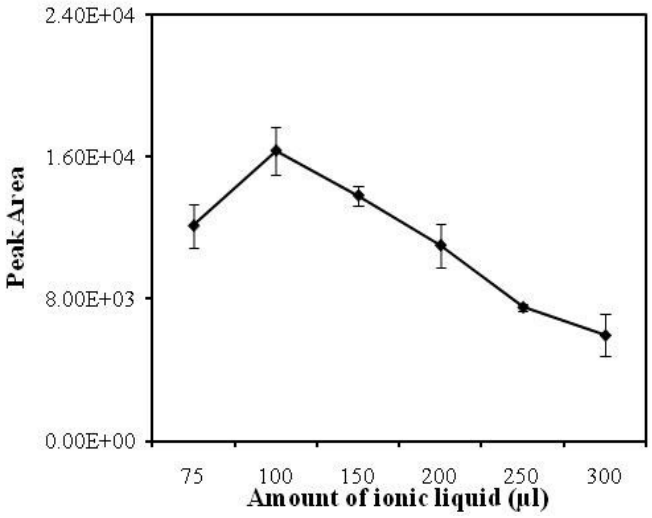


Figure 4.4: Volume of ionic liquid (5 mL reaction mixture containing spiked MCLR (50 µg L^{-1}) with 75-300 µL of BMiM PF_6 maintained at pH 3; incubated for 10 at 65ºC. Ionic liquid phase injected in LC/MS/MS water (0.1% formic acid): methanol: 90:10; 0.3 mL min^{-1}).

As the extraction and pre-concentration involves the extraction of MCLR from the aqueous phase into the much condensed ionic liquid rich phase, it is envisioned that the detection limits can be lowered with a larger sample volume. Therefore, the effect of sample volume on the extraction efficiency was also studied keeping the concentration of ionic liquid constant as obtained from above mentioned set of experiments. It was found that 5 mL of the sample volume gave the highest peak area corresponding to the maximum extraction efficiency (Figure 4.5). Upon increasing the volume above 5 m (65°C; 10min), the mixture did not form emulsion upon rapid cooling, which could be a

possible reason for low extraction efficiency at higher volumes. Moreover, it was found that as the sample volume was increased, the phase separation between the ionic liquid and water decreased. The recovery of the ionic liquid markedly decreased which resulted in less extraction efficiency. The low recovery of the ionic liquid upon cooling from the emulsion could be attributed to solubility characteristics of BMiM PF_6 which were discussed in the earlier section. For further experiments, 5 mL was therefore used for extraction.

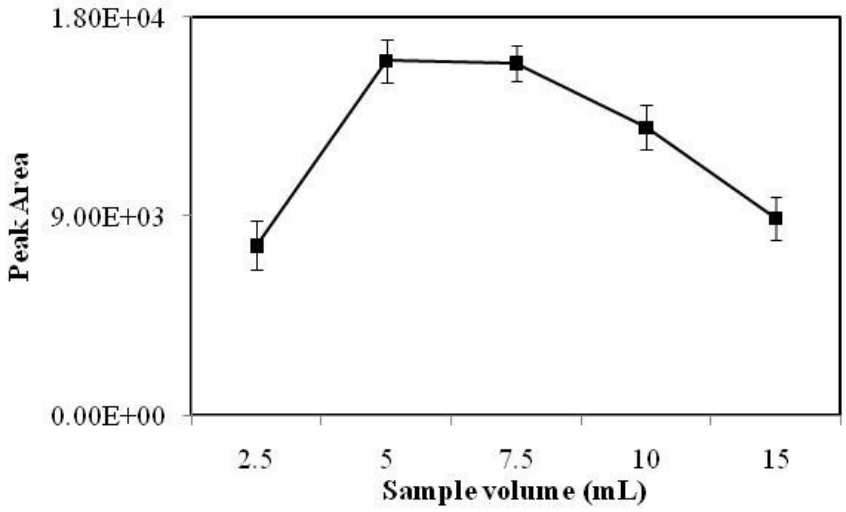


Figure 4.5: Extraction volume (Different volumes of reaction mixtures were prepared spiked with MCLR (50 µg L^{-1}) with 100 µL of BMiM PF_6 maintained at pH 3; incubated for 10 at 65ºC. Ionic liquid phase injected in LC/MS/MS water (0.1% formic acid): methanol: 90:10; 0.3 mL min^{-1}).

4.3.4 Quantitative information

To assess the practical applicability of the proposed method, the optimized extraction conditions were adopted to evaluate performance characteristics such as linearity, LOD and limit of quantification (LOQ) for MCLR and MCRR. Lowest detection limit (LOD) is the lowest quantity of a substance that can be distinguished from the absence of that substance (a blank value) within a stated confidence limit (generally 1%). Limit of quantification (LOQ) is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of test. A signal-to-noise ratio (S/N) of three is generally accepted for estimating LOD and signal-to-noise ratio of ten is used for estimating LOQ. This ratio was used in current experiments to arrive at LOD and LOQ for the developed analytical method.

5 mL of nanopure water spiked with known concentrations of MCLR, MCRR (50 µg L^{-1}) and 100 µL of ionic liquid was kept in a water bath at 65 °C for 10 min. After incubation, it was rapidly cooled in water at 4°C for 2-3 min and then centrifuged at 4000 rpm for 5 min. Linearity was investigated from 0.5 to 50 µg L^{-1} by plotting the LC-MS-MS peak areas of MCLR/MCRR against the concentrations in the spiked nanopure water samples. The least square linear regression was used to analyze the linearity.

Table 4.1

Linearity range of calibration plot, LOD, LOQ and precision (%RSDs) of extraction method

Analyte	R.S.D % (n=6)	Regression equation	Linearity range ($\mu g\ L^{-1}$)	LOD (μgL^{-1})	LOQ (μgL^{-1})	Correlation coefficient
MCLR	7.5	Y = 384.24x + 260.01	0.5 - 50	0.03	0.15	0.9954
MCRR	7.2	Y = 1068x - 1162	0.5-50	0.09	0.45	0.997

The correlation coefficients were determined to be 0.995 and 0.997 at 95 % confidence level which are acceptable for trace analysis. Thus, a proportional relationship can be calculated between the amount of analytes extracted and the concentration of the sample. The LOD was determined by progressively decreasing the concentration of MCLR/MCRR spiked in the water sample until distinct responses were still clearly observed at a signal to noise (S/N) ratio of 3 (n=6). The LOD thus estimated was 0.03 and 0.09 $\mu g\ L^{-1}$ for MCLR and MCRR respectively. The LOQ was determined at S/N =10, which was 0.15 and 0.45 $\mu g\ L^{-1}$ (Table 4.1) for MCLR and MCRR respectively. The low LOQ value is sufficient to measure the concentration of MCLR in environmental water samples.

The LOD obtained from this study was compared to those reported in other studies (see Table 4.2). The proposed LOD value was superior to those reported for other micro- extraction techniques such as SPME (Poon et al., 2001) and cloud point extraction method with cationic surfactant (Man et al., 2002). The method developed by Murata et al., has lower LOD

for MCRR (0.03 µg L- 1) as compared to our study. However, for MCLR (0.03 µg L- 1) the LOD observed by them is the same as the LOD observed in our study. SPE-HPLC-UV method by Lee et al., 1999 achieved better LOD (0.02 µg L- 1 for MCLR and MCRR) than obtained in the present study. However, SPE (solid phase extraction) employs toxic organic solvents like methanol for elution and concentration of MCs. The present method was aimed at developing greener technologies for extraction and preconcentration of MCs. Although there are differences in LOD, the present method is an alternative to the conventional methods that are cumbersome and not environment friendly.

These differences in LOD could be due to sensitivity of the method for particular MCs. Since the present method is based on polarity and ion pairing between analyte and the ionic liquid, some MCs could be more likely to get extracted as compared to others. Based on their interaction with ionic liquids, their LOD would be affected. However, this LOD is fairly good as WHO safety guidelines for MCs in drinking water are 1 µg L- 1 of MCLR/ MCLR equivalents. The present method can still be used for routine monitoring.

Table 4.2
Performance of ionic-liquid supported cloud point extraction with other methods in literature

Quantification method	Method Detection limit MCLR /MCRR µg L^{-1}	Reference
Soncation-LC-MS-MS2	0.2/0.2	Spoof et al., 2003
Filtration-UPLC-MS-MS	0.06/0.06	Xu et al., 2008
SPE-HPLC-UV	0.02/0.02	Lee et al., 1999
Derivatization-HPLC-Chemiluminescence	0.03/0.03	Murata et al., 1995
Organic modifiers-CE/UV	0.12/0.12	Onyewuenyi et al., 1996
SPE-CE/MS	200/NA*	Bateman et al., 1995
SPME-HPLC-UV	7.4/7.1	Poon et al., 2001
Cloud point extraction-HPLC/UV	0.15/NA*	Man et al., 2002
Present study (Cloud point extraction-LC-MS-MS)	0.03/0.09	-

**NA: not reported*

4.3.5 Application to real environmental water samples

The developed extraction/pre-concentration method was applied to real environmental water samples collected from local reservoirs. The physiochemical properties of the water samples are presented in Table 4.3.

Table 4.3

hysiochemical Properties of the samples collected from three different sites of a natural reservoir located in Singapore.

Physiochemical Properties	**Site 1**	**Site 2**	**Site 3**
pH	7.71	7.61	6.21
Salinity (parts per thousand)	0.04	0.04	0.88
Conductivity (mS cm^{-1})	0.067	0.068	1.649
Turbidity (NTU)	1.2	0.3	83.0
Chlorophyll (%)	1.9	1.8	67.7
Dissolved oxygen (DO) %	123	126	54.6

Significant differences were observed in the physiochemical properties of water samples collected from sites 1 and 2 when compared to site 3. Water quality parameters such as salinity, conductivity, and cholorophyll counts were higher in the case of the sample from site 3 as compared to others. The percentage recoveries of MCLR/MCRR at 10 µg L^{-1} spiked in reservoir water samples ranged from 83.7 to 92.5 % in the water samples (Table 3.4).

Table 4.4

Percentage recoveries of MCLR (spiked at 10 μg L^{-1}) using ionic-liquid supported cloud point extraction from the real samples collected from three different sites.

Sample	Concentration detected in samples MCLR/MCRR (μg L^{-1})	Amount detected (μg L^{-1})	Percentage Recovery MCLR/MCRR
Site 1	ND	8.36±0.95	83.66
		8.64 ±0.83	86.4
Site 2	ND	8.55 ± 1.10	85.50
		9.06 ± 0.76	90.6
Site 3	6.2 / 9.2	15.45 ± 1.34	92.5
		18.2 ± 0.98	90.0

The recovery of organic species from water samples depends upon the salinity, TOC (Total organic content) and pH of the sample matrix. Studies have shown that trace organic species have strong affinity for organic matter which could contribute to lowered recovery in the samples analyzed. However, in this case major matrix interferences were not observed. Therefore, the method is suitable for analyzing water samples with a range of physiochemical profiles. In water samples collected from site 3, 6.2 and 9.2 μg L^{-1} of MCLR and MCLR was detected respectively (Figure 4.6).

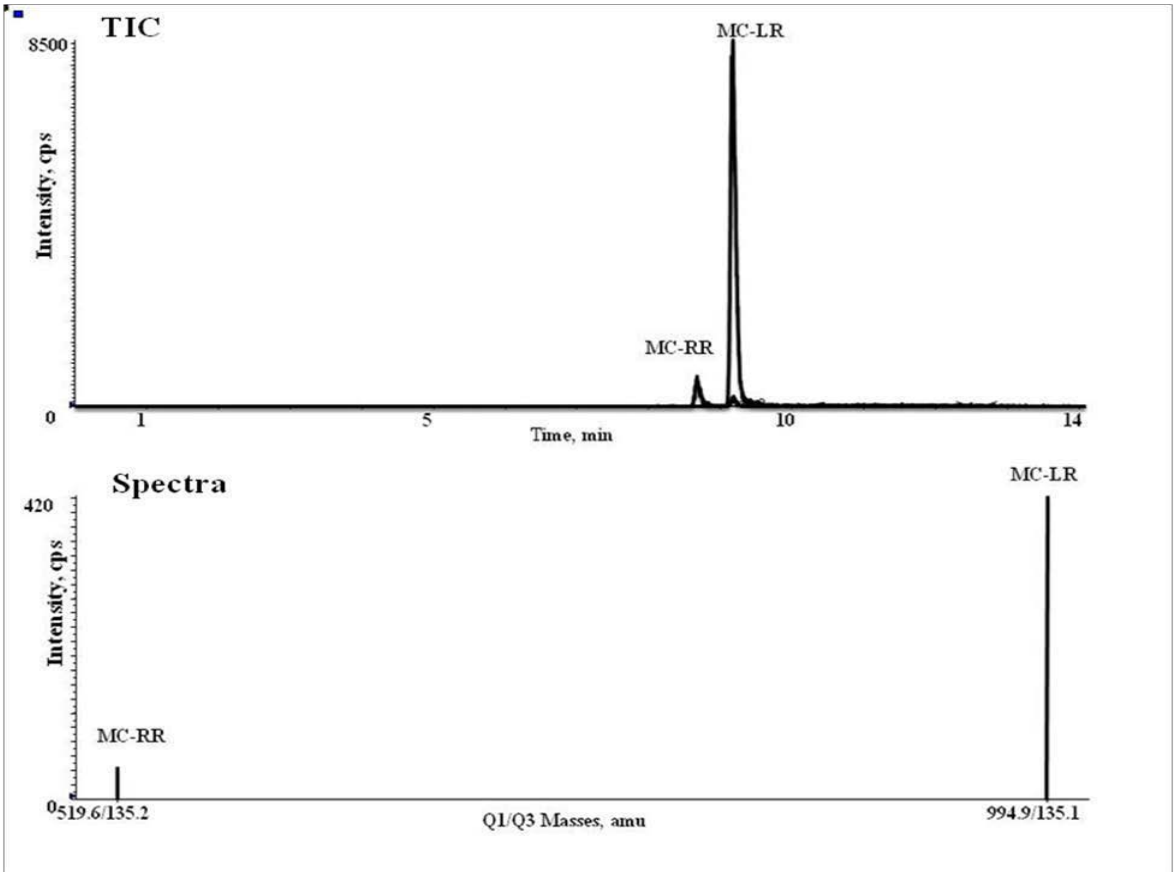


Figure 4.6: LC-MS-MS chromatogram of spiked real water extract (site 3). 5 mL of site 3 water sample spiked MCLR/MCRR at **(**10 µg L^{-1}) with 100 µL of BMiM PF_6 maintained at pH 3; incubated for 10 min at 65 ºC. Ionic liquid phase injected in LC-MS-MS (RP Zorbax C_{18} 0.1% formic acid: methanol: 90:10; injected sample volume 10 µL; 0.3 mL min^{-1}).

The present analytical method is simple, efficient, and environmentally friendly. It could be used as a routine monitoring tool for analyzing MCLR and MCRR in natural water systems.

4.4 Conclusion

The optimized ionic liquid extraction technique developed and used in this study in tandem with LC-MS-MS meets the requirement of being sensitive, efficient and environmentally benign. It can be used for identification and quantification of MCLR and MCRR in environmental water samples. This novel extraction technique has the potential to be used for the routine analysis of water samples containing MCLR and MCRR and possibly other MCs for which further work is needed.

CHAPTER 5

BIOCHEMICAL CHANGES IN ZEBRAFISH ORGANS UPON A BALNEATION EXPOSURE

This chapter describes the biochemical changes in zebrafish organs, namely, gills, liver, intestine and brain following a balneation exposure to MCLR/MCRR. Five concentrations (0.1 µg l^{-1} to 10.0 µg l^{-1}) were selected for the exposure. The zebrafish were divided into five experimental groups exposed to five different concentrations of MCLR/MCRR. The zebrafish were sampled out after 5, 7 and 15 days of exposure. After sampling, their tissues were harvested and the activity of biochemical enzymes was measured. This was done to (i) understand the dose-response relationship of MCLR/MCRR when administered under balneation conditions (ii) understand the toxicological implications of MCLR/MCRR at low, sub-chronic doses to zebrafish.

5.1 Introduction

Toxic cyanobacterial blooms have been reported frequently worldwide, and their occurrence has caused severe problems to wildlife, livestock and humans (Codd et al., 1997; Hudnell, 2010). The intracellular toxins are often released into the water bodies following an episode of bloom lysis, or an algicide treatment (details in Chapter 2, section 2.2). The presence of MC with a range of concentrations (1 to 141 µg L^{-1}) has been reported in the water phase at different time points from different regions in the world (Christoffersen, 1996; Izaguirre et al., 2007; Jones and Orr, 1994; Te and Jin

2010). Most aquatic organisms can come in contact with the released toxins, or with the cyanobacterial cells, leading to possibility of their accumulation in the aquatic food web (as discussed in Chapter 2, section 2.5). It has been shown that certain groups of organisms (e.g. protozoa, rotifers) are highly sensitive to these toxins, which implies that processes such as microbial loop are disturbed with consequences for other organisms in the food web (e.g. fish, crustaceans) (Christoffersen, 1996; Fulton and Pearl, 1987; Nizan et al., 1986). Fish have been used in a number of studies to evaluate the health impact of microcystin toxicity. There have been studies on zebra fish embryos, *Corydorus paleatus* (cat fish), *Cyprinus carpio* (common carp), *Hypophthalmichthys molitrix* (silver carp) and few other species either injected intraperitoneally with MCLR or MCRR, or exposed to a cyanobacterial bloom (Cazenave et al., 2006a; Wiegand et al., 1999). Biochemical, histopathological, and behavioral patterns in the fish have been subsequently examined on exposure to microcystins (Blaha et al., 2004; Cazenave et al., 2006b; Li et al., 2005). However, the uptake and severity of effects vary largely depending upon the dose of these toxins and the exposure route.

In real aquatic ecosystems, organisms would often be exposed to trace levels of toxins persisting in water for long time periods following a lysis episode (Jones and Orr, 1994). These conditions would lead to a balneation exposure i.e. aquatic organisms would be bathed in the water containing the trace levels of dissolved toxins. In order to make realistic evaluation on MCs toxicity, chronic and sub-chronic exposure routes should be thoroughly studied. Exposure routes and dosage are very important parameters for any experimental design (Ron et al., 2003). Studying the possible exposure routes would offer a deep understanding of bioaccumulation and uptake pathways involved in the

toxicity of MCs. Organisms often adapt to the stress situations and respond differently under different conditions. Therefore, there is a great need to explore all the possible pathways through which aquatic organisms are exposed to MCs for complete risk assessment and risk management in water bodies.

The *in vivo* metabolism of these toxic compounds often leads to the formation of reactive oxygen species (ROS), which significantly contribute to their toxicity (Ding et al, 2001; Ding et al., 2000; Li et al., 2003). The reduction products of molecular oxygen such as hydroxyl, superoxide and peroxyl radicals are of great significance, as they may react with critical cellular macromolecules, possibly leading to enzyme inactivation, DNA damage, lipid peroxidation and ultimately cell death (Zegura et al., 2008; Zegura et al., 2003). Generation of ROS is an inevitable part of aerobic life. However, organisms have evolved with diverse mechanisms to combat the effect of the normal flux of ROS generated by respiration, certain enzyme activities, and phagocytosis (Harmann 1992). These mechanisms include enzyme systems from glutathione family, superoxide dismutases and catalases that act to remove ROS (biochemical defenses), low molecular weight compounds that directly scavenge ROS and proteins that sequester prooxidants such as transition metals. Together, these mechanisms form the antioxidant defense systems (Hudson, 1990). Antioxidant defenses can be altered in response to exposure to oxidative stress, or increased ROS in a variety of ways (As discussed in Chapter 2, section 2.6).

Alteration of biochemical defense systems is typically the initial response to any toxic exposure and therefore the measurement of these systems can be sensitive indicators of altered cell function. Consequently, biochemical defenses can serve as biomarkers of

interest, and can be used as a component for risk assessment (Ron, 2003). These enzymes systems, though highly sensitive, are not selective and specific to the kind of insult faced by the organism. The activities of these enzymes would vary considerably depending upon the dosage and time of exposure to the concerned organism. Moreover, continual exposure to low levels of toxins can confer resistance to the organisms and subsequently can adapt their systems to the stress observed by a variety of ways. To understand the response or toxicological implications to an organism in a realistic way, it would be important to study the changes at various time points. Single time points would elucidate the toxic potency of the particular MC of concern, but would not offer any information on adaptability of the organism in question to the situation.

The present study has attempted to evaluate the effects of sub-lethal (i.e. dosage below which lethality is not observed) concentrations of MCLR and MCRR (extracellular) dissolved in water on adult zebra fish using biochemical biomarkers at three time points. Four enzymes, namely, superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione-S-transferase (GST) were investigated as biomarkers for oxygen mediated toxicity. These antioxidant enzyme responses were studied in liver, gills, intestine and brain tissues of zebra fish exposed to dissolved MCLR and MCRR (0.1 µg L^{-1} to 10.0 µg L^{-1}).

5.2 Methodology

5.2.1 Fish exposure and enzyme measurement

Chemicals, reagents used for these experiments are given elsewhere in Chapter 3 (section 3.2.1, 3.2.2). Details about selection and procurement of fish are given out in Chapter 3

(section 3.4.1 and 3.4.2). For schematic on experimental design of exposure for these studies, refer to Figure 3.3 (Chapter 3, section 3.3.3).

Fish were exposed to MCLR and MCRR dissolved in aquarium water at concentrations of 0.1, 0.5, 1.0, 5.0 and 10 µg L^{-1} for a period of 15 days. Briefly, stock solutions of MCLR and MCRR were prepared (10 mg L^{-1}) and diluted to obtain the required concentrations. Water in the tanks was replaced with fresh water spiked with the toxin daily. Dosing concentrations were confirmed by measurements using liquid chromatograph, composed of an HP100 liquid chromatograph (Agilent Technologies, U.S.A) interfaced with a triple quadrupole MS/MS (Applied Biosystems, U.S.A). Analytical separation was achieved on a Zorbax Extend-C18 5 µm, 2.1 x 150 mm (Agilent technologies, Germany). The injection volume was 10 µL. The mobile phase consisted of 0.1 % formic acid (solvent A) and methanol (solvent B). A gradient elution was used, starting with water: methanol at 90:10 from 0 to 6 min, and switching to 5:95 up to 10 min before returning to the original conditions to re-equilibrate the system.

The capillary voltage was set at 89 volts and the cone voltage at 4 volts. The desolvation gas (nitrogen) temperature and flow-rate were set at 350°C and 615 L/h, respectively. The ion source temperature was set at 120°C. LC-MS-MS was operated in the positive ion mode. MCLR and MCRR were monitored by using the MS instrument in the SRM mode: m/z 995.6; fragment ion at 135.1 and m/z 520; fragment ion 135.1, respectively.

Fish were sampled out at 4 days (n=8), 7 days (n=8) and 15 days (n=8). Enzyme measurements were carried out for all the time points. A control tank was also maintained for 15 days under the same conditions without MCLR or MCRR. After

exposure, fish were sacrificed and dissected. Gills, liver, intestine and brain from control and exposed animals were separated, snap frozen in liquid nitrogen and stored at -80°C until measurements. For biochemical enzyme measurements, tissues were homogenized using 50 mM potassium phosphate buffer, pH 6.5 containing 1 mM EDTA. After removal of cell debris (14,000 g x 10 min, 4^{o}C), the resulting supernatant was used for the enzyme activity measurement. For enzymatic measurements four kits were used namely GST (EC.2.5.1.18), GPx (EC 1.11.1.9), GR (EC 1.6.4.2) and SOD (EC 1.15.1.1). Details on the purchase and working principle of the kits are given elsewhere in Chapter 3 (Section 3.5.2 and 3.6.1). Estimation of enzymes in the tissue homogenates was done using UV-plate reader (Chapter 3, section 3.6.1). The enzymatic activity was calculated in terms of the protein content of the sample and is reported in nmol/ml/min/mg protein (Chapter 3, section 3.6.1).

5.2.2 Statistical analysis

All chemical and biochemical results are reported as "Mean ($\bar{x}$) ± Standard deviation (σ)" of eight animals per group per time point. Data were analyzed using Graphpad Prism 5 (Version 5.0 for Windows, CA, USA). All the data were first examined visually and statistically for normality of distribution. A two-way ANOVA was performed to assess the significance of response in relation to concentration, time and possible interactions of these factors on the response.

5.3 Results and Discussion

It was observed that the swimming behavior was normal among fish until 7 days of exposure to MCLR and MCRR. However, when exposed to higher concentrations of

MCs ($\geq$ 5.0 µg L^{-1}), the zebra fish showed disturbances in their swimming behavior. Some of them showed decreased motilities, but no mortality was observed. Similar responses were observed in a previous study in which the highest dose of MCLR used decreased the swimming activity of the exposed fish (Baganz et al., 2004; Cazenave et al., 2008).

For the first enzyme studies (GST), there was a decrease in the enzyme activity of the exposed fish at the first time point (4 days) for 0.1 µg L^{-1} dosage of MCLR as compared to controls. However, for higher concentrations of MCLR, the enzyme activity was higher than that of the control with a peak at 0.5 µg L^{-1} in the case of the intestine, but there was no significant change beyond 1.0 µg L^{-1}. For other tissues, a peak in the enzyme activity was also observed at 0.5 µg L^{-1}, but there was a decrease in the activity beyond 1.0 µg L^{-1}. However, for MCRR exposed groups, the GST activity in all tissues was higher at lower concentrations as compared to the control, but was lower at concentrations $\geq$ 5.0 µg L^{-1}. At the second (7^{th} day) and the third time points (15^{th} day), the enzyme activity for MCLR exposed tissue groups was higher in the case of liver and brain (up to 1.0 µg L^{-1}), but was lower in the case of intestine and gills (all concentrations except 0.1 µg L^{-1}) as compared to the first time point. In the case of MCRR exposed tissue groups, the enzyme activity was higher for all dosage groups at both second and third time points with respect to that observed at first time points in all the tissues (Figure 5.1 and 5.2). Results obtained from the exposure experiments showed that both the dose of MCLR/RR and the duration of exposure had a significant impact on the enzyme (GST) activity associated with tissues of zebra fish. Moreover, the interaction between the dose and the duration of exposure was also significant, and

contributed to the change in the enzyme activity observed in the tissues. The significance levels observed as an effect of dose, time and their interaction is $p < 0.0001$ for tissues (Figure 5.1 and 5.2).

The GPx activity showed a wide variation in terms of its enzymatic activity for the three time points studied (Figures 5.3 and 5.4). At the first time point, the GPx activity had its peak at 1.0 µg L^{-1} in liver following exposure to MCLR and in liver and intestine as for MCRR exposure. For other tissues exposed to MCLR and MCRR, a concentration of 0.5 µg L^{-1} showed the highest amount of the enzyme activity.

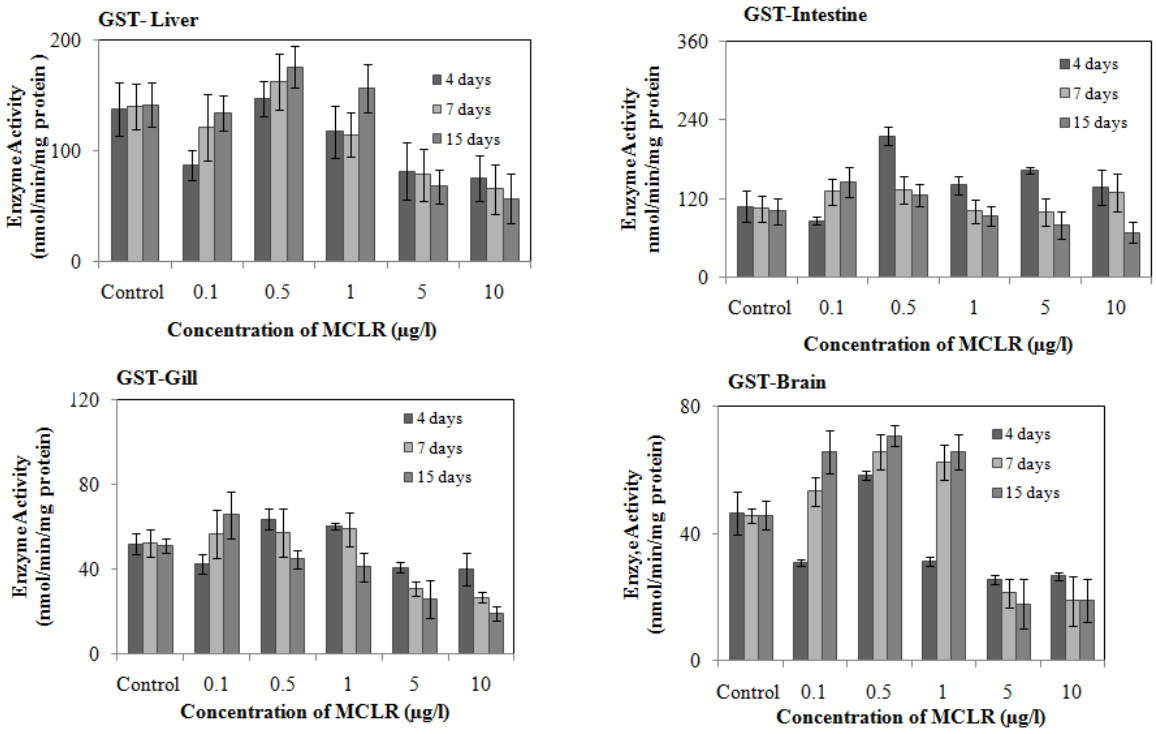


Figure 5.1: GST activity in liver, intestine, gills and brain of zebra fish exposed to MCLR at different time points. The values are expressed as $\bar{x} \pm \sigma$

However, exposure to concentrations above 1.0 µg L^{-1} showed a decline in enzyme activities for both MCLR and MCRR with respect to those observed at lower concentrations. After 7 and 15 days of exposure, for MCLR, the enzyme activity did not show any consistent pattern in response to changes in the dose except that it had its peak at 1.0 µg L^{-1} in liver and gills. Interestingly, for MCRR, the enzyme activity showed its maximum value at 1.0 µg L^{-1} in liver and intestine tissue and at 0.5 µg L^{-1} in gills and brain. In general, the enzyme activity decreased at concentrations higher than 1.0 µg L^{-1} among all exposed tissues. The statistical analysis showed no significant impact of exposure duration (MCLR) on the GPx activity in gills. However, for all other tissues, the dose of MCLR/RR, the duration of exposure and their combined effect showed a significant impact on the GPx activity in zebra fish tissues. The significance levels observed in case of MCLR treated tissues as an effect of dose are $p < 0.001$ (Liver [L] , Intestine [I] , Gill [G]), $p < 0.0058$ Brain [B]; as an effect of time are $p < 0.001$ (L, I, B); as an effect of interaction between time and dose are $p < 0.001$ (I, B), $p < 0.006$ (G), $p < 0.0029$ (L). The significance levels observed in the case of MC-RR treated tissues as an effect of dose are $p < 0.001$ (L, I, G, B) ; as an effect of time are $p < 0.001$ (L,G,B), $p < 0.0042$ (I) ; as an effect of interaction between time and dose are $p < 0.001$ (I,B,G,L).

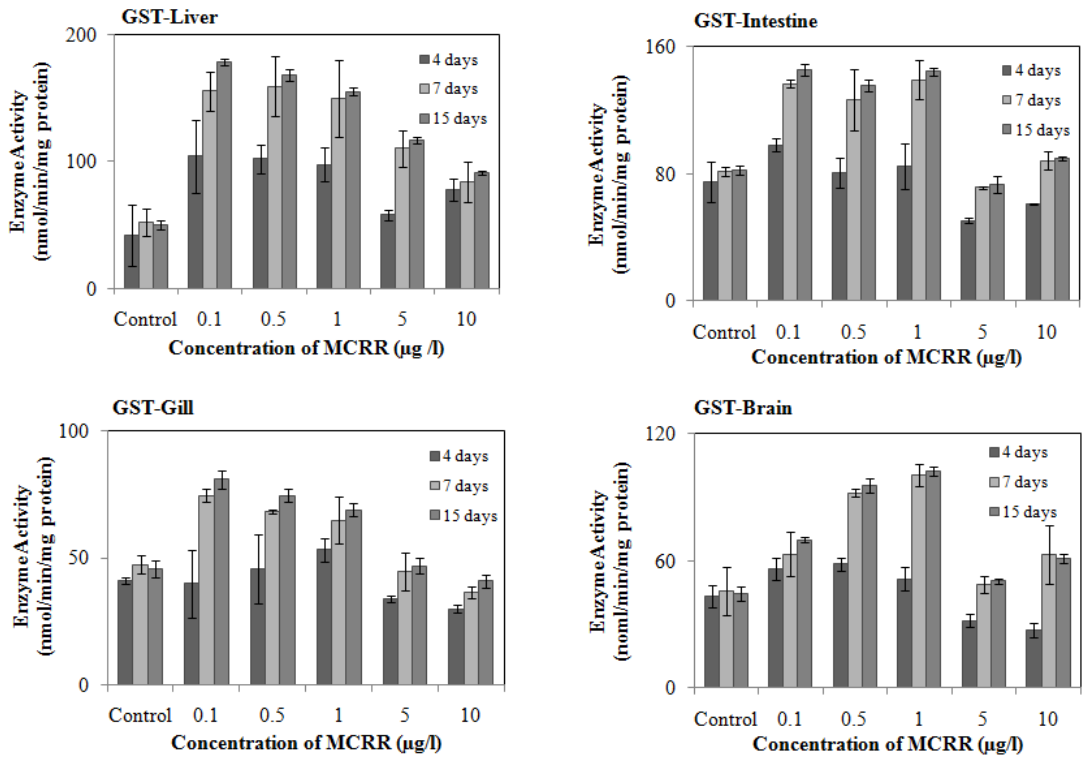


Figure 5.2: GST activity in liver, intestine, gills and brain of zebra fish exposed MCRR at different time points. The values are expressed as $\bar{x} \pm \sigma$

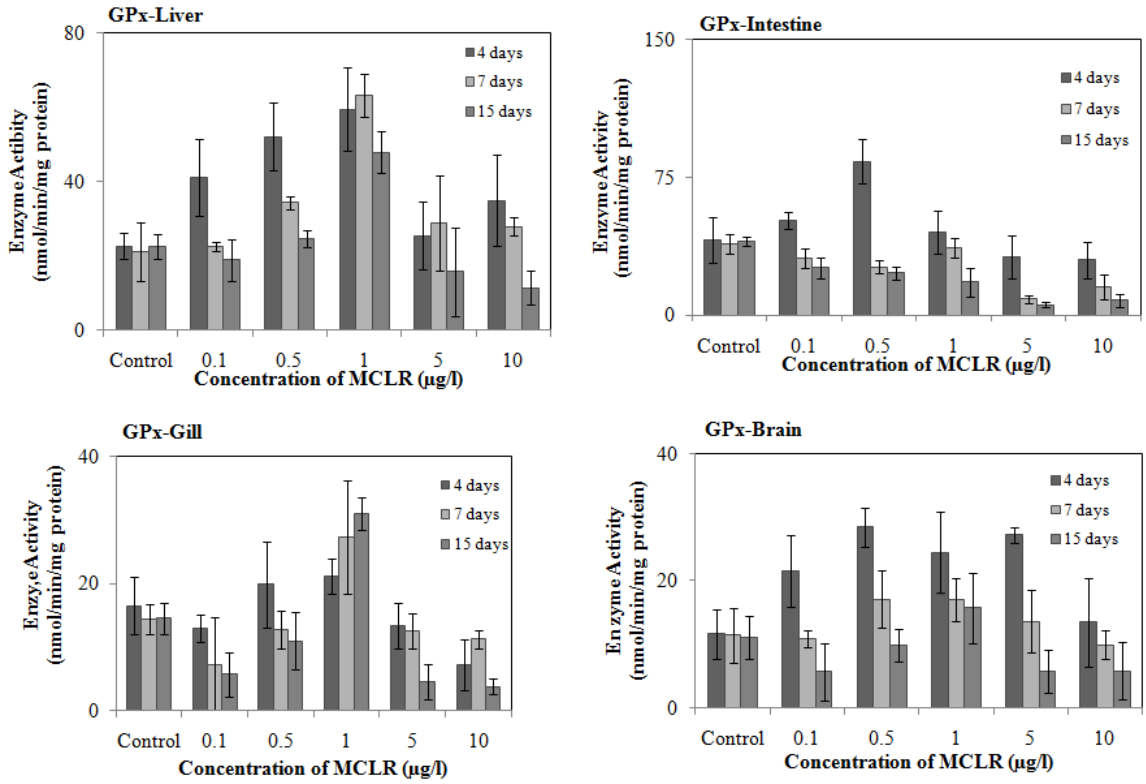


Figure 5.3: GPx activity in liver, intestine, gills and brain of zebra fish exposed to MCLR at different time points. The values are expressed as $\bar{x} \pm \sigma$

GR activity, for the first time point (4 days), increased significantly in all tissues at ≥ 1.0 µg L^{-1} for fish exposed to MCLR (Figure 5.5). However, for MCRR, the GR activity increased as compared to controls among low concentrations, reaching its peak at 1.0 µg L^{-1} for liver and gills and at lower concentrations for intestine and brain tissues (Figure 5.6). For second (7 days) and third (15 days) time points in the study, no general trend was observed in the enzyme activities for MCLR exposed tissue groups. For MCRR, there was an increase in enzyme activities in general for the experimental groups studied as compared to controls at the second time point (7 days) in all tissues except gills where there was no consistent pattern. Interestingly, a peak was observed in liver at 5.0 µg L^{-1},

in intestine at 1.0 µg L^{-1} and 10.0 µg L^{-1} for the second time point. At the 15th day, there was a further increase in enzyme activities in intestine and brain tissues at the exposure dose of 0.1 and 0.5 µg L^{-1}, respectively.

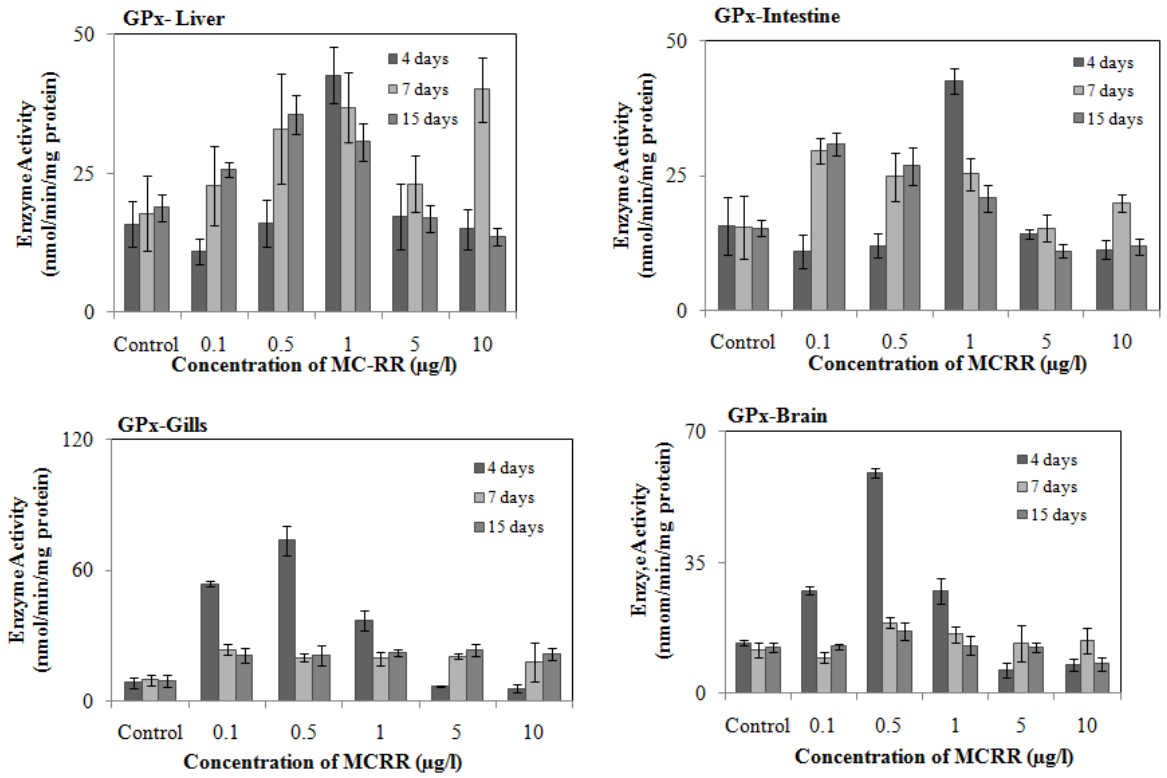


Figure 5.4: GPx activity in liver, intestine, gills and brain of zebra fish exposed to MCRR at different time points. The values are expressed as $\bar{x} \pm \sigma$

Statistical analysis of data revealed that exposure to MCLR had significant impact on the GR activity in all tissues except liver. In the case of MCRR, there was no significant effect of dose on the GR activity in the liver and gills. Likewise, there was no significant effect of exposure duration on the GR activity in the gills and brain. The significance

levels observed in case of MCLR treated fish as an effect of dose are $p < 0.001$ (I, G, B); as an effect of time are $p < 0.001$ (I, G), $p < 0.002$ (B); as an effect of interaction between time and dose are $p < 0.012$ (G), $p < 0.0001$ (I, B). The significance levels observed in case of MCRR treated fish as an effect of dose are $p < 0.0001$ (I, B); as an effect of time are $p < 0.0001$ (L, I); as an effect of interaction between time and dose are $p < 0.017$ (L), $p < 0.0028$ (I), $p < 0.0022$ (G) (Figure 5.5 and 5.6).

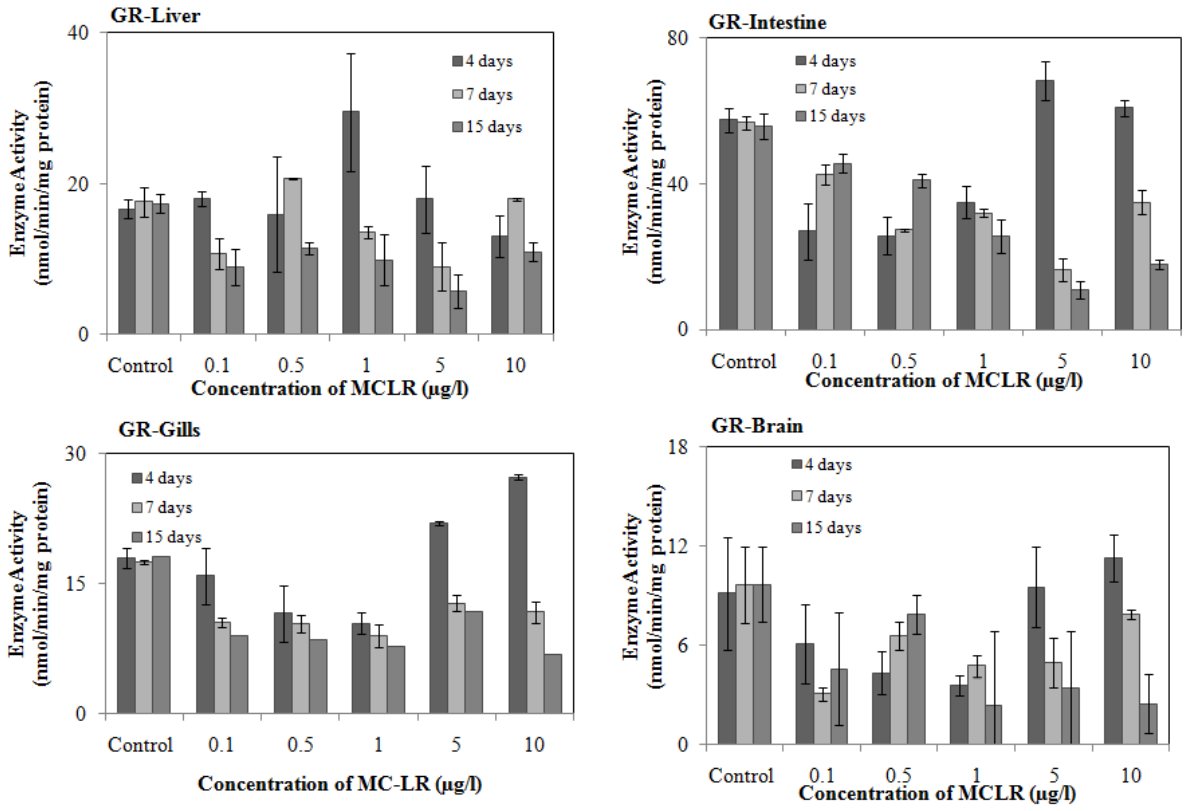


Figure 5.5: GR activity in liver, intestine, gills and brain of zebra fish exposed to MCLR at different time points. The values are expressed as $\bar{x} \pm \sigma$

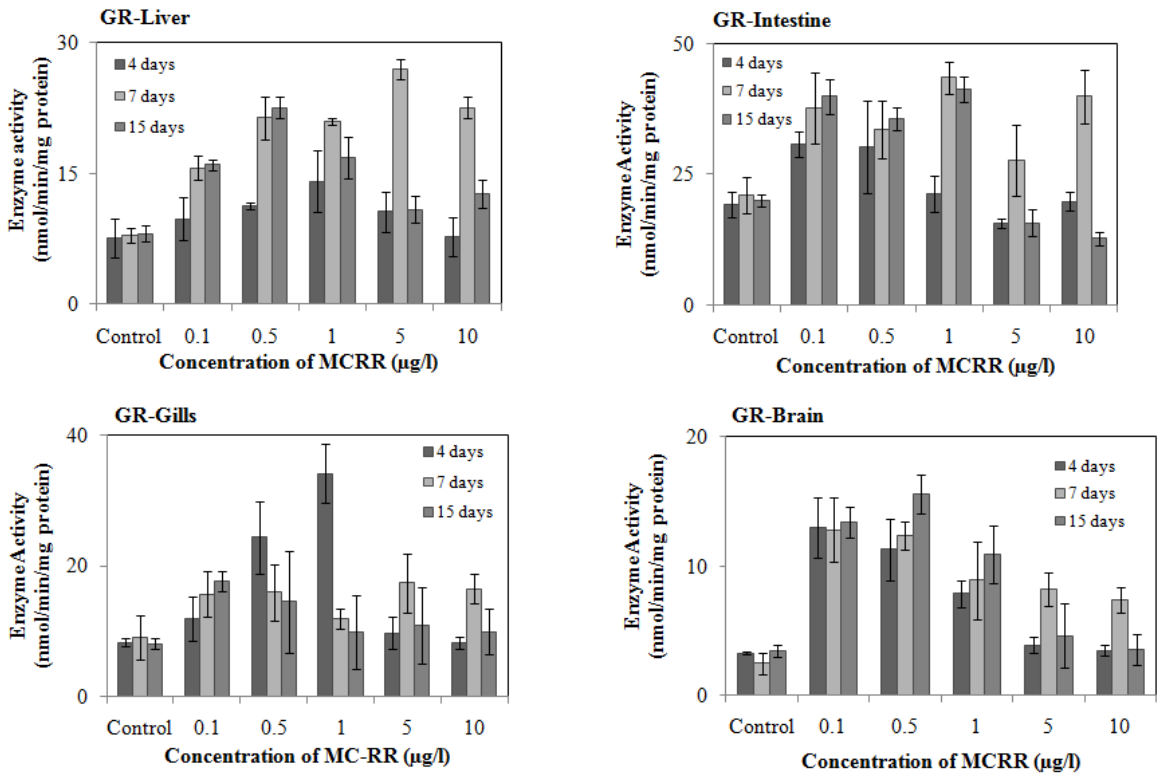


Figure 5.6: GR activity in liver, intestine, gills and brain of zebra fish exposed to MCRR at different time points. The values are expressed as $\bar{x} \pm \sigma$

The SOD activity (measured in terms of % inhibition) increased at lower concentrations as compared to controls , and decreased at concentration ≥ 5.0 µg L^{-1} for the first time point (4 days) for MCLR (Figure 5.7) . For MCRR, the enzyme activity first decreased in all tissues except liver as compared to controls, attained a peak subsequently and then decreased at concentration ≥ 5.0 µg L^{-1} (Figure 5.8). However, the enzyme activity increased after 7 days of exposure for MCLR exposed tissues at concentrations ≤ 1.0 µg L^{-1}. A similar trend was observed after 15 days for MCRR exposed tissues except the brain. Results show that there was a significant effect on enzyme activities resulting from different doses (MCLR/RR), exposure length and the interaction of dose and time

among all tissues. The significance levels observed in case of MCLR exposed fish as an effect of dose are $p < 0.0032$ (I) $p < 0.0001$ (L, B, G); as an effect of time are $p < 0.032$ (I) as an effect of interaction between time and dose are $p < 0.0001$ (L), $p < 0.0002$ (I), $p < 0.0003$ (G), $p < 0.0206$ (B).

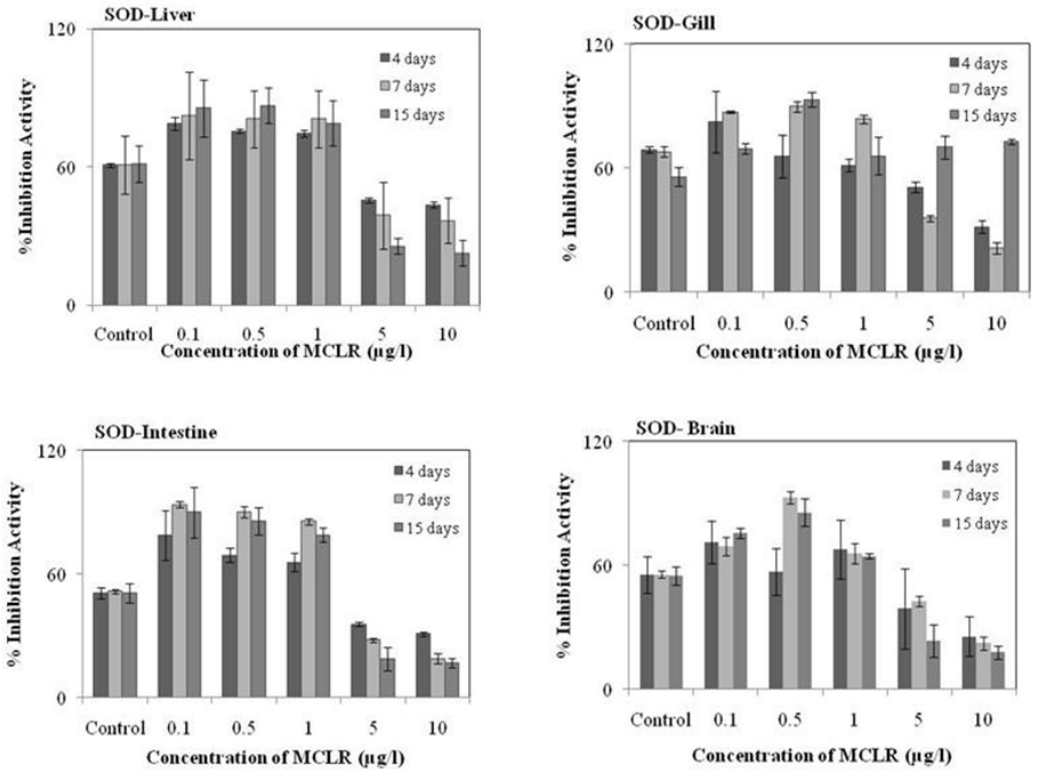


Figure 5.7: SOD activity in liver, intestine, gills and brain of zebra fish exposed to MCLR at different time points. The values are expressed as $\bar{x} \pm \sigma$

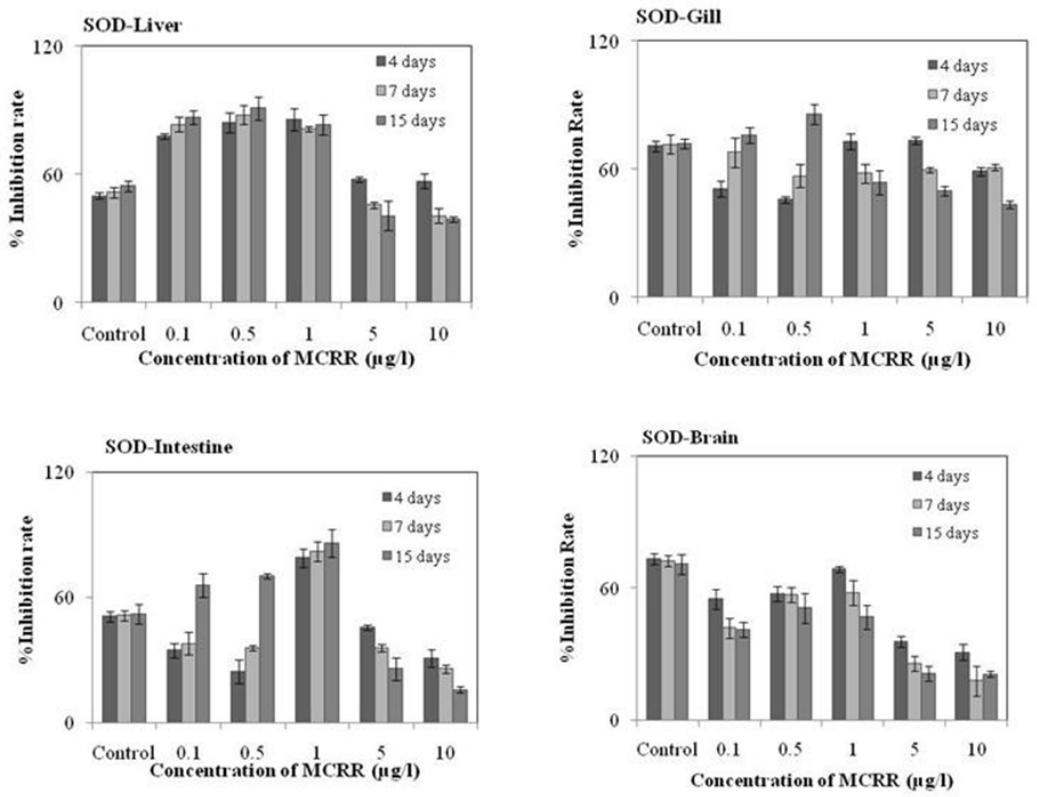


Figure 5.8: SOD activity in liver, intestine, gills and brain of zebra fish exposed to MCRR at different time points. The values are expressed as $\bar{x} \pm \sigma$

The significance levels observed in the case of MC-RR exposed fish as an effect of dose are $p < 0.0001$ (L, I, G, B); as an effect of time are $p < 0.001$ (L, I, G) $p < 0.0035$ (B) as an effect of interaction between time and dose are $p < 0.0001$ (L, I, G) (Figure 5.7 and 5.8).

Exposure to MCLR and MCRR dissolved in water in sub-lethal concentrations induced perturbations in the in the antioxidant and detoxification enzymes activities for most of the dosage groups under study (For example Figure 5.3 and 5.4), It was observed that enzyme activity reached its peak at a particular concentration in different dosage groups

suggesting a trigger in the molecular mechanisms of enzyme synthesis (de novo synthesis), or activation of existing enzymes. Further, at high concentrations, the enzyme activity was inhibited in most of the cases observed for both MCLR and MCRR (Figure 5.1 and 5.2). This suppression in the enzyme activity could be attributed to secondary effects due to substrate inhibition. In most of the groups, this trend was observed for the first time point. Enzyme activity blockage resulting from inhibition has been explained in recent studies conducted by other research groups with a variety of hypotheses (Egaas et al., 1999; Gallangher and Sheehy, 2000). It is suggested that inhibition of an enzyme due to substrate may be characterized by a covalent modification of the enzyme leading to an irreversible loss of activity. Alternatively, lower activities of enzyme may be caused by a decrease in the synthesis of the proteins at the molecular level (Egaas et al., 1999; Gallangher and Sheehy, 2000).

When the fish were continually exposed to MCLR and MCRR for more than four days, it was also observed that some antioxidant enzymes showed an adaptive response at second and third time points, wherein the enzyme activities increased in some tissues (for example gills in case of SOD; liver and intestine in case of GR) following sustained exposure to MCLR/RR. There are no published reports on acquired resistance to MC toxicity by aquatic organism to date. However, in the literature there are reports on cells derived from Chinese hamster acquiring resistance on exposure to low levels of hydrogen peroxide, leading to better cell survival. The antioxidant enzyme activity was shown to increase in those cells upon exposure to low levels of cytotoxic drugs (Koyeli et al., 2005). It was suggested that cells can respond to oxidative stress by induction of antioxidant enzymes (Christova et al., 2003; Franco et al., 1999). The synthesis of

antioxidant enzymes could be a plausible explanation for the increase in enzyme activity following MC exposure in some experimental groups.

The family of GST enzymes forms the first detoxification pathway after exposure to MCs. As reported by Pflugmacher et al (1998), the GST enzyme catalyzes the conjugation of MCs with glutathione in several aquatic organisms (Pflugmacher et al., 1998). Moreover, the conjugate formed is soluble in water and can thus be eliminated from the fish body. Metcalf et al. (2000) showed that the conjugate of MCLR and MCYR with both cysteine and glutathione is less toxic than MCs alone, which confirms the hypothesis that conjugation catalyzed by GST is the first step to detoxification (Metcalf et. al., 2000). The conjugate formation implies that GST has an important role to play when MCs are released from cyanobacteria followed by their uptake by aquatic organisms. An elevation in GST activity thus suggests that an increased conjugate formation between GST and MCs and subsequent elimination of MCs are likely to take place. On the other hand, a decrease in the enzyme activity could potentially result in altered biochemical effects in the organisms exposed to MCs, leading to bioaccumulation accompanied by cellular damage and physiological, behavioral changes (Baganz et al., 2004; Malbrouck et al., 2003). In the present study, an increase in GST activity was observed for certain organs at the 7^{th} and 15^{th} day, which is suggestive of adaptive response by the tissues in response to continued exposure to toxins) (Figure 5.1 and 5.2). This adaptive response is indicative of a natural defense mechanism by zebra fish to avoid bioaccumulation of MCs since the GST can conjugate MCs with glutathione and thus eliminate the toxins from the body. However, no previous study has reported the change in GST upon exposure to MCs over a time point range, so results obtained from

this study could form a basis for further in-depth research on the formation of the MCs-GST conjugates and their role in detoxification.

Wiegand et al. (1999) reported a dose-dependent increase in the GST activity over ontogenetic development for zebra fish embryos (from 0.1 to 2.0 µg L^{-1} MCLR). However, it was observed that a higher dose suppressed the GST activity (Wiegand et al., 1999). Cazenave et al. (2006a) also reported higher GST and CAT activities with 25 µg L^{-1} of MC-RR and MC-LF (Microcystin- Leucine-Phenyl alanine) dosing in REKO medium for embryos (Cazenave et al., 2006a). The GST activity observed in this study is consistent with the findings reported by Wiegand et al. (1999); Cazenave et al. (2006a) on embryos. This agreement suggests that the biochemical response of adult fish is quite similar to that of the embryos with respect to the GST profile. This finding can be particularly helpful in studying the adverse effects of MCs on organisms across the ecological webs over generations. Moreover, in this study, a dose dependent increase in the GST activity was observed over the three time points studied. However, at concentrations of MCLR and MCRR $\geq$ 5.0 µg L^{-1}, there was a decrease in the enzyme activity resulting from enzyme inhibition which could be explained by secondary effects as discussed earlier in this section. Inhibition of the GST activity has also been reported in microalgae, macrophytes and fish eggs exposed to cyanobacterial extracts (Pietsch et al., 2001).

Another balneation study with a similar experimental design was recently reported by Cazenave et al. (2006b), in which *Corydoras paleatus* (cat fish) was exposed to MCRR up to 10.0 µg L^{-1} for a period of 24 hours (Cazenave et al., 2006b). According to this

study, a dose-dependent reduction of the GST activity was observed in both liver and brain of exposed fish at high concentrations of MCRR. However, an elevation in the GST enzyme activity was noticed in the gills and intestine of the exposed fish at high concentration of MCRR. Similar results were obtained in present study where the enzyme activities decreased at higher concentrations of MCRR at first time point. However, the results obtained in this present study differed from those from other studies in which the GST activity was either unaffected by oral exposure to cyanobacterial extracts (Gehringher et al., 2004; Leão et al., 2008; Malbrouck at el., 2003), or increased at the highest dose (1 µg g^{-1} oral feed with MCRR) as reported by Cazenave et al. (2008) . These different results could be explained in terms of species-specific biochemical behavior towards MCLR/RR.

Another endogenous antioxidant defense mechanism includes the family of GPx and GRs. Peroxidases aid in combating oxidative stress by detoxifying hydrogen peroxide and fatty acid peroxides (Arthur, 2000). The principal peroxidase in fish is a selenium-dependent tetrameric cytosolic enzyme that employs GSH as a cofactor. GPx catalyzes the metabolism of hydrogen peroxide to water, involving a concomitant oxidation of reduced glutathione (GSH) to its oxidized form (GSSG). GR is not involved in the antioxidant defense directly as GPx does, but it merits attention because of its importance in maintaining GSH/GSSG homeostasis under oxidative stress conditions (Winston and Di Giulio, 1991). GR catalyzes the transformation of GSSG to GSH with the concomitant oxidation of NADPH to $NADP^{+}$. In the present study, there was a wide variation observed in terms of enzyme activities across various time points. Moreover, enzyme response differed between MCLR and MCRR at the same time point in the same

tissue. This difference in response from MCs could be explained by their toxicity potentials. Despite being more toxic, the adaptive response of MCLR is delayed. In contrast, MCRR is less toxic than MCLR, but it makes a rapid adaptive response at the second time point (7^{th} day) onwards as can be seen in the profile of GR (Liver, intestine and gills).

Results obtained from the present study are consistent with those reported by Wiegand et al. (1999) on *zebra fish* embryos where GPx activities were increased in embryos exposed to 0.5 µg L^{-1} MCLR (Wiegand et al., 1999) as compared to controls. A similar pattern was observed for other fish species wherein the GPx activity was induced on exposure to MCLR (10 µg L^{-1}) in hepatocytes of common carp (Li et al., 2003), in liver and kidney of tilapia fed with cyanobacterial cells (containing MCLR) during 21 days (Jos et al., 2005). Cazenave et al. (2006a) studied the GPx activities after exposure of zebrafish embryos to MCRR, and did not observe any change as compared to controls which is in contrast to the findings of the current study (Cazenave et al., 2006a). However, few other studies reported that MCRR showed an induction of the GPx activity on uptake through different routes (Cazenave et al., 2006b; Prieto et. al, 2007). At later stages i.e. at 7 days and 15 days, an opposite trend was observed in the GPx response for MCLR and MCRR exposed fish (Figure 5.3 and 5.4). More detailed investigations are needed to understand the difference in responses among the different organs of fish at different time points following exposure to MCs. These differences in response could play an important role when risk assessment of any water body is desired since most of the times one kind of microcystins dominates during the bloom episode. Moreover, since MCLR and MCRR could be present concurrently following a cyanobacterial bloom, the

uptake kinetics of MCLR and MCRR by different organisms of fish could provide insightful information into changes in their corresponding enzyme activity.

GR also showed great variations in response among the concentrations studied for MC-LR and MCRR as was observed for GPx (Figure 5.3 and 5.4). A plausible explanation to this similarity in the dose-reponse relationship could be the close relationship between the two enzymes. In addition, the GR activity is influenced by the availability of reducing equivalents such as NADPH. In the case of oxidative stress, glucose is directed to the pentose phosphate pathway to generate NADPH needed for this reaction (Worthington and Rosemeyer, 2005). However, glucose and glycogen metabolic pathways are regulated by a group of protein phosphatases and are dependent on these enzymes for activation/deactivation for certain rate limiting enzymes (DeMott and Dhawale, 1995). Since protein phosphatases are irreversibly inhibited by MCs (DeMott and Dhawale, 1995), there could be some irregularities observed in these metabolic pathways as well. As a consequence, there could be improper channeling, or shuttling of glucose and its metabolites. This improper shuttling of metabolites could affect the pentose phosphate pathway which utilizes glucose-6-phosphate, subsequently disturbing NADPH production and GR status in the organism. However, this hypothesis needs to be experimentally verified before drawing any realistic conclusions.

From literature, Cazenave et al. (2006a) reported no effect on the GR activity on exposure to MCRR (Cazenave et al., 2006a). However, another study reported a decrease in the GR activity on exposure to MCRR (Prieto et al., 2007) while two other studies reported an increase in the GR activity on exposure to MCLR (Cazenave et al., 2006;

Joset. al., 2005). In general, there was a wide variation in the response observed across different fish species and even among studies with different exposure routes for different time frames. For risk assessment and management, as discussed earlier, a case-to-case basis approach should be taken into consideration owing to the wide differences observed by the various research groups who studied the biochemical changes in fish following exposure to microcystins.

SOD activity was also investigated by some authors. Recent studies by Atencio et al. (2008) and Prieto et al. (2007) reported a decrease in the SOD activity after exposure to MCLR by *Tinca tinca* (doctor fish) (oral exposure to cyanobacterial cells dosing 5, 11, 25 and 55 mg MCLR/fish) and by *Oreochromis niloticus* (nile tilapia) (food pellets with 1350 µg MCLR/g of pellet), respectively (Atencio et al., 2008; Prieto et al., 2007). However, all other studies reported an increase in the SOD activity on exposure to MCs (Jos et al., 2005; Li et al., 2005; Prieto et al., 2007). In the present study, both an increase and a decrease in the SOD activity were observed for MCLR. However, for MCRR, a reverse pattern was observed (Figure 5.7 and 5.8). Prieto et al. (2006) also observed a difference in response to oxidative stress caused by MCLR and MCRR (ip) in *Oreochromis sp.* (nile tilapia) (Prieto et al., 2006). These observations further demonstrate the differential response by zebra fish to oxidative stress caused by MCLR and MCRR. It would be interesting to evaluate the underlying biochemical mechanisms responsible for the difference in responses observed by different research groups. As mentioned earlier, the difference in responses between MCLR and MCRR are likely due to the toxicity potentials of both the variants which could in turn influence the nature of the response by the cells and tissues.

5.4 Conclusion

This study provides insights into the biochemical marker (oxidative stress) response elicited by MCLR and MCRR in adult zebra fish under balneation conditions. It explains the possible adaptive behavior of the zebra fish with continued exposure to low levels of MCLR and MCRR as experienced under natural conditions. The findings of the study improve our understanding of the impact of the MCLR and MCRR dissolved in the water (extracellular) column on the aquatic life species under natural conditions when exposed for a sustained period of time. It shows that even under non lethal conditions, MCLR and MCRR can cause deleterious effects when present in extracellular forms.

This fundamental knowledge provides a framework for exposure and impact assessment of microcystins based on which effective management of water resources can be further developed. However, since biochemical markers are not specific to the kind of toxicity faced by an aquatic organism, experiments are warranted at metabolic and molecular levels to understand the plethora of implications. Additionally, experiments are also needed to understand the extent of bioaccumulation of MCLR and MCRR. This would help in understanding the uptake of MCLR and MCRR by various tissues. The present preliminary data provides the input for further in-depth studies as discussed in later chapters.

CHAPTER 6

IMMUNOHISTOCHEMISTRY FOR LOCALIZED BIO-DISTRIBUTION OF MCLR AND MCRR IN ZEBRAFISH

This chapter describes the examination of thin tissue sections obtained from zebrafish after a balneation exposure to MCLR/MCRR at 10 µg L^{-1} for a period of 30 days. Following the exposure, the tissue samples from zebrafish were processed to obtain thin tissue sections for microscopy. After obtaining the tissue sections, they were stained with a specific primary antibody for MCLR/MCRR. The primary antibody binds to MCLR/MCRR present in the tissues due to its high affinity for these MCs. The tissue sections were subsequently incubated with a secondary antibody. The secondary antibody binds to the primary antibody and generates a signal which is a colured chromophore and can be monitored under a normal inverted microscope. The intensity of the colored chromophore is directly proportional to the amount of MCLR/MCRR present in the tissue site. These experiments were done to detect the presence of MCLR/MCRR in tissues of zebrafish.

6.1 Introduction

Histological examinations (light microscopic study of thin tissue sections) have been used by the scientific community to elucidate the toxicological implications of a variety of environmental contaminants (Pacheco and Santos 2002; Husoy et al., 1996). For understanding the toxicokinetics and mechanisms of MCs toxicity, scientists have used histological procedures such as different kinds of staining techniques to elucidate the

changes that occur in tissues and cells following expsore of fish to MCs (Philips et al., 1995; Tencalla et al., 1994). Apart from determining the extent and mechanism of damage caused by toxins, histological techniques can also be used to understand the localization of the contaminants in various organ systems of the test organisms to study their biodistribution within fish. This could further aid in evaluating the extent of exposure to chemical contaminants which can then serve as biomarkers of exposure. Bioaccumulation markers and biomarkers of exposure reflect the distribution of the chemicals or their metabolites, respectively, throughout the organism (WHO, 1993). One of the histological procedures to determine the localisation of toxins and environmental contaminants in tissues is Immunohistochemistry (IHC).

IHC is a valuable tool for the identification and visualization of tissue antigens in biological research and clinical diagnostics. IHC can characterize various biological processes (Aikawa 2011). In brief, it refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues (Ramos-Vara, 2005). There are limited reports on the localization of MCs using immunohistochemical procedures. Lance et al. (2010) and Djediat et al. (2010) used immunohistochemistry to show localization of MCLR in medaka fish and gastropoda, respectively (Lance et al., 2010; Djediat et al., 2010). The results obtained from any histological experiment would vary depending upon the length and route of exposure of fish or any test organisms to the toxins. Results would also depend upon the species in question, as some species could be more toxic than the others and might show an immediate response in terms of tissue and cellular structure disruption following an exposure episode. Hence, for a deep understanding of

toxicokinetics originating from different routes of exposure, there is a need for further research in this area. Previous studies reported on immunohistological examinations done on tissues exposed to MCs were restricted to acute exposures mainly through intraperitoneal or oral gavage route (Lance et al., 2010; Djediat et al., 2010).

An attempt was made in this doctoral study to understand the pattern of localization that can be observed in the zebrafish tissues upon exposure to extracellular MCLR and MCRR (under chronic balneation conditions).

6.2 Methodology

6.2.1 Fish exposure and sampling

Chemicals, reagents used for these experiments are given elsewhere in Chapter 3 (section 3.2.1, 3.2.2). Details about selection and procurement of fish are given out in Chapter 3 (section 3.4.1 and 3.4.2). For schematic on experimental design of exposure for these studies, refer to Figure 3.4 (Chapter 3, section 3.3.4). Fish were exposed to MCLR and MCRR dissolved in aquarium water at a concentration of 10 µg L^{-1} for a period of 30 days. This concentration was decided based on the outcomes of the previous experiments conducted in this study (concentrations of MCLR/MCRR found in real water samples: 6.2-9.2 µg L^{-1}; biochemical systems of zebrafish organs largely affected at highest dose i.e. 10 µg L^{-1}- Chapter 4 and 5). Briefly, stock solutions of MCLR and MCRR were prepared (10 mg L^{-1}) and were diluted to obtain the required concentrations (Chapter 3, section 3.2.4). Water in the tanks was replaced with fresh water spiked with the toxin daily. Dosing concentrations were confirmed by measurements using a liquid chromatograph, composed of an HP100 liquid chromatograph (Agilent Technologies,

U.S.A) interfaced with a triple quadrupole MS/MS (Applied Biosystems, U.S.A). Analytical separation was achieved on a Zorbax Extend-C18 5 µm, 2.1 x 150 mm (Agilent technologies, Germany). The injection volume was 10 µL. The mobile phase consisted of 0.1 % formic acid (solvent A) and methanol (solvent B). A gradient elution was used, starting with water: methanol at 90:10 from 0 to 6 min, and switching to 5:95 up to 10 min before returning to the original conditions to re-equilibrate the system (As done for biochemical experiments, chapter 5).

The capillary voltage was set at 89 volts and the cone voltage at 4 volts. The desolvation gas (nitrogen) temperature and flow-rate were set at 350°C and 615 L/h, respectively. The ion source temperature was set at 120°C. LC-MS-MS was operated in the positive ion mode. MCLR and MCRR were monitored by using the MS instrument in the SRM mode: m/z 995.6; fragment ion at 135.1 and m/z 520; fragment ion 135.1 respectively.

At the end of exposure period, fish (n= 4) were sampled out and they were slit open ventrally from the heart to the anus to expose the digestive organs. The entire fish was fixed in a 10% formalin solution for 3-4 days. Fixed fish samples (n=4 per group; MCLR, MCRR and control fish) were washed several times with 70% ethanol, followed by dehydration in a graded series of ethanol before clearing in Histoclear and embedding in paraffin. The paraffin embedded tissues were sectioned serially at 5-µm thickness and used for immunohistochemical study (Details in Chapter 3, section 3.5.3).

6.2.2 Immunohistochemistry procedures

For immunohistochemistry of paraffin sections, antigen retrieval was performed by heating before treating sections with 6% hydrogen peroxide in methanol for peroxidise

blocking and in blocking buffer (10% bovine serum-PBS) for 20 min to block nonspecific binding. Primary antibody namely a monoclonal antibody to MCs (ADDA specific; AD4G2) was incubated at room temperature for 4 h (Alexis Biochemicals, Switzerland). Rabbit anti-mouse antibody conjugated with horseradish peroxidase (Zymax grade, DAKO) for detection by DAB substrate-chromagen (DAKO) was used as a secondary antibody. Negative controls were set up by omitting primary antibodies. Positive reactivity was revealed by the formation of brown-colored precipitate at the site of the target antigen (Details in Chapter 3, section 3.5.3).

6.2.3 Microscopic evaluations

Sections were lightly counterstained with hematoxylin. Images of the stained liver, gills and intestine sections were captured using Axiovert microscope (Zeiss) equipped with an imaging system

6.3 Results and Discussion

In order to localize covalently bound MCLR and MCRR in zebrafish tissues, four individuals, and five tissue sections per individual, from each exposure group were immunohistochemically stained for MCs and assessed after the exposure. Control zebrafish sections showed a negligible amount of background and thus false-positive MC-immunostaining (Figure 6.1, 6.2 and 6.3), while zebrafish exposed to either MCLR or MCRR showed a strong (significantly higher than the background signal) MC-immunopositive staining. MC-immunopositive staining was observed in gills, intestine and liver of the exposed zebrafish sections.

Anti-microcystin labeling with antibody AD4G2 was visible in the livers of treated animals (MCLR and MCRR) as dispersed positive foci with staining surrounding the hepatocytes, and also accumulated in the sinusoid capillaries (Figure 6.2). Irregular immunopositive staining was detectable in the intestinal (Figure 6.1) and gill tissue (Figure 6.3) of the fish exposed to MCLR and MCRR.

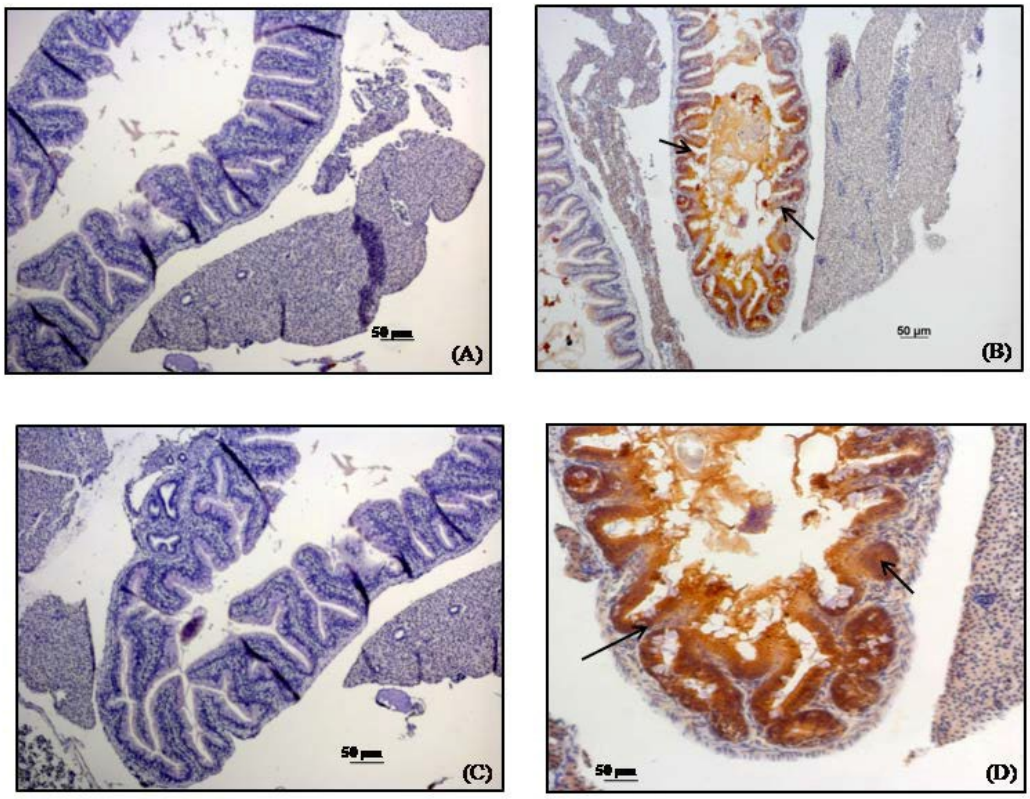


Figure 6.1: Semi-thin section of adult zebrafish intestine anti-microcystin labeling (AD4G2) with hydroxyperoxidase. A) Control: There is no labeling in the submucosa B) Treated (30 d after balneation episode with MCLR). Positive hydroxyperoxidase anti-microcystin labeled areas are detectable in the intestine submucosa (arrowheads). C) Control: Details of the submucosa, the cells are not labeled. D) Positive hydroxyperoxidase anti-microcystin (MCRR) labeled areas can be seen in the cytoplasm of the submucosal macrophages.

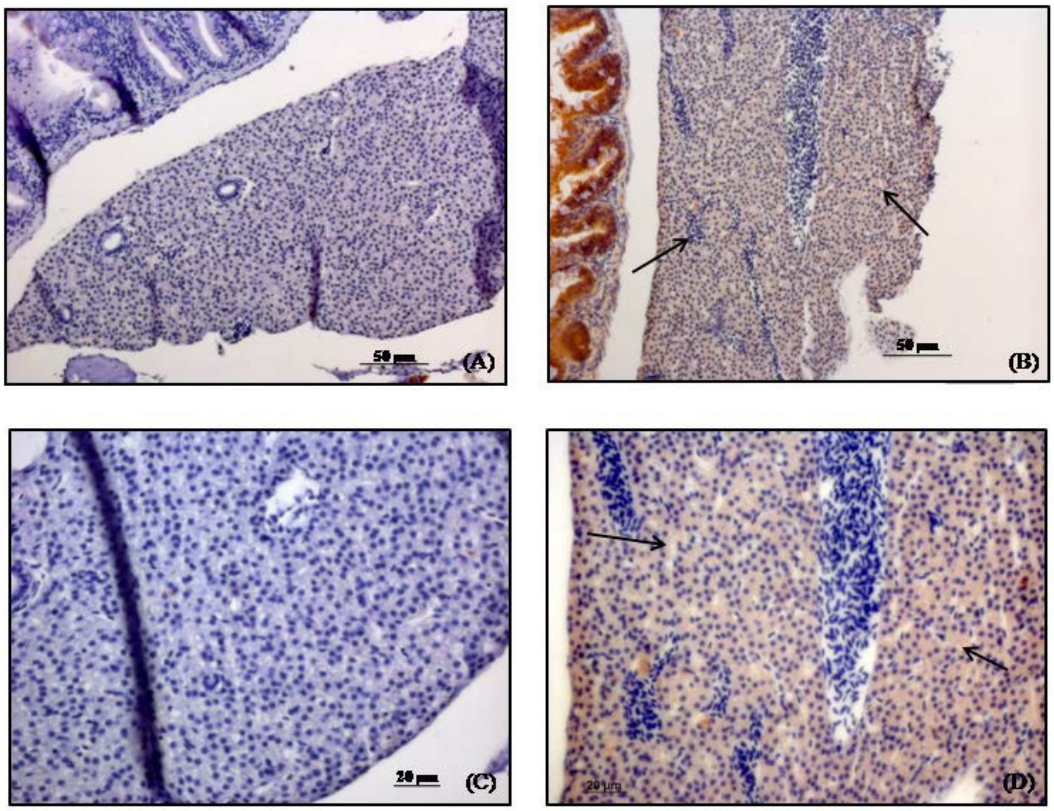


Figure 6.2: Semi-thin section of adult zebrafish liver, anti-microcystin labeling (AD4G2) with hydroxyperoxidase. A) Control (water with no MCLR or MCRR). B) Treated (30 d after balneation exposure with MCLR, the brown color corresponds to positive hydroxyperoxidase anti-microcystin labeled areas (arrowhead) C) Hepatocytes of a control fish, with no positive staining observed; D) Hepatocytes of treated zebrafish (MCRR), with positive hydroxyperoxidase anti-microcystin labeled areas (arrows).

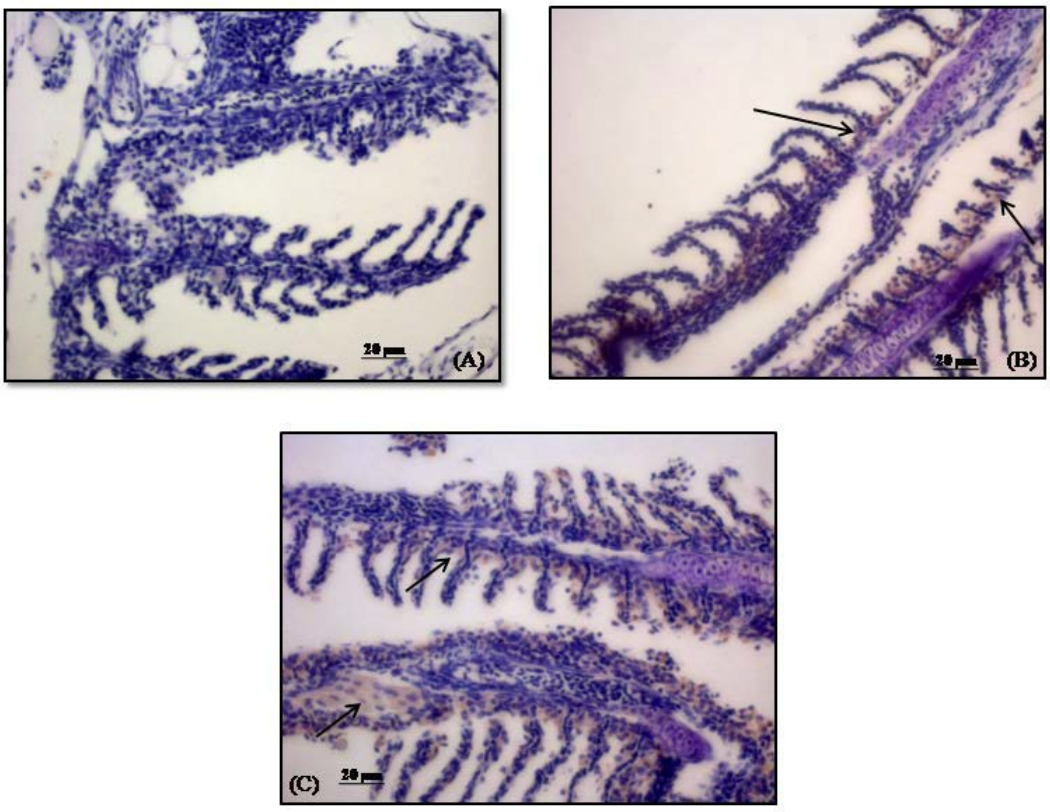


Figure 6.3: Semi-thin section of adult zebrafish gill, anti-microcystin labeling (AD4G2) with hydroxyperoxidase. A) Control (water with no MCLR or MCRR) B) Treated (30 d after balneation exposure with MCLR, the brown color corresponds to positive hydroxyperoxidase anti-microcystin labeled areas (arrowhead) C) Gill section of treated zebrafish (MCRR), with positive hydroxyperoxidase anti-microcystin labeled areas (arrows).

It is clear from the figures presented above that MCLR and MCRR have indeed been taken up by various tissues in zebrafish. Balneation route of exposure would mainly cause the toxin uptake via gills (inhalation) and through dermal surface (Evans 1987). It should be noted that this route of exposure implies very active absorption through the gills and distribution in the body by-passing the first-pass metabolism. Normally, the first-pass effect (also known as first-pass metabolism or presystemic metabolism) is a phenomenon whereby the concentration of a chemical species is greatly

reduced before it reaches the systemic circulation. It is the fraction of lost chemical compounds such as toxicants, drugs and other substances during the process of absorption which is generally related to the liver and gut wall (Pond and Tozer 1984). After a chemical compound or toxicant is ingested, it is absorbed by the digestive system and enters the hepatic portal system. It is carried through the portal vein into the liver before it reaches the rest of the body (Giulio and Hinton 2008). The liver metabolizes many drugs and toxins; sometimes to such an extent that only a small amount of active chemical component emerges from the liver to the rest of the circulatory system. This *first pass* through the liver thus greatly reduces the bioavailability of the chemical species.

The first pass usually takes places when the exposure route is oral. In the present study, fish exposure to toxins is mainly through dermal (via skin) and inhalation (via gills), in which case liver is by-passed, MCs are taken up by the pulmonary and systemic circulation and then distributed to the different organ systems including liver (Figure 6.4). Liver is a main detoxifying organ for MCs since liver contains GSH which is known to complex with MCs and form soluble complexes which can then be eliminated via excretory routes (Pflugmacher et al., 1998). Since liver is the major detoxification organ for MCs, any exposure route that by-passes liver for metabolism would thus increase the bioavailability of MCs to other organs. Increased bioavailability could have serious toxicological implications under chronic exposure to different organ systems depending upon the biodistribution of MCs.

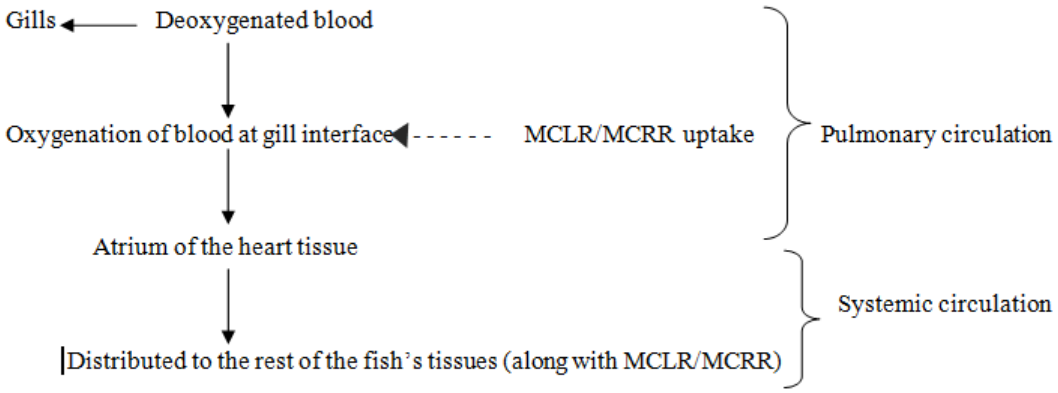


Figure 6.4: MCLR/MCRR distribution and uptake kinetics in zebrafish tissues for present study

In the present study, strong anti-microcystin labelling was found in all the tissues including liver, gills and intestine (Figure 6.1-6.3). However, the first organ of interaction has been gills; the staining has been found in other tissues as well. In fish it is assumed that the main routes of access to the body are through the gills and through the gut via the food (Lien and McKim, 1993). A counter-current mechanism in fish gills provides a potentially efficient transport route for the uptake of chemicals such as MCs from water into the bloodstream (Hayton and Barron, 1990). In fish, the oxygenated blood travels from the gills directly to most organs, including the gonads. Therefore, MCs entering the bloodstream in this way go directly to the target organs, before any degradation can occur, a process predominantly mediated in the liver as mentioned in the previous section. In the present exposure, there is likelihood that MCLR and MCRR could have been taken up along with the oxygenated blood and exchanged via pumps with deoxygenated blood in a counter current system. The oxygenated blood containing

MCLR and MCRR could then possibly reach the heart tissue, and from heart it could be distributed to different organs. If the exposure route was oral, absorbed MCLR and MCRR from gastrointestinal (GI) tract would first reach liver where detoxification can take place, from liver, it would enter the systemic circulation and reach the heart (Giulio and Hinton, 2008)

Although balneation episode may not result in fatality, it could still affect a lot of organ systems in long term thus affecting the entire ecosystem. This makes balneation route of exposure very important and distinct from other common routes of exposure such as ingestion wherein a large amount could be lost in liver (Giulio and Hinton, 2008). Therefore, this exposure route cannot be evaded while designing a risk assessment and management policy.

6.4 Conclusion

From the immunochemistry experiments done in this study, it is clear that although gill and dermal tissues were exposed, both MCLR and MCRR were biodistributed in intestine and liver as well through the systemic circulation. Preliminary experiments showing biochemical response also support the results obtained from qualitative biodistribution experiments, wherein oxidative stress response was observed in all the above mentioned tissues including gills. Although oxidative stress was also observed in brain tissues, positive anti-microcystin labelling was not found in brain sections exposed to either MCLR or MCRR. The absence of positive anti-microcystin labelling in brain could be due to low concentrations of MCLR and MCRR (less migration of MCLR and MCRR

across the blood brain barrier) in brain tissue which could not generate enough signals when labelled with an anti-microcystin antibody.

Bioaccumulation of MCLR and MCRR in tissues can further perturb the metabolic pathway functioning in these organs. This bioaccumulation could also affect the tissue at molecular or gene levels, causing up/down regulation of transcripts. It is, therefore, of paramount importance to study the metabolites and transcripts of these tissues under the conditions known to cause accumulation in order to understand the implications of the accumulated MCLR and MCRR in zebrafish.

CHAPTER 7

METABOLIC CHANGES IN ZEBRAFISH ORGANS UPON A BALNEATION EXPOSURE

This chapter describes the in-depth toxicological changes that occurred in zebrafish tissues after the exposure to MCLR/MCRR dissolved in water. After the exposure period, four tissues namely, gills, intestine, liver and brain were harvested from the zebrafish. After harvesting the tissue, small molecules called metabolites were extracted using different solvents following a sequential extraction procedure. These metabolites arise from a number of biochemical pathways and processes occurring in zebrafish organ systems. Upon an exposure to a toxicological agent, biochemical pathways required for the normal growth and development of zebrafish may get disrupted. This disruption can be identified through the evaluation of metabolites. In this experiment, attempt was made to evaluate the extent and mechanism of toxicity caused by MCLR/MCRR to zebrafish by monitoring the metabolites.

7.1 Introduction

Uptake of environmental pollutants and toxicants by aquatic organisms such as fish has been associated with adverse health effects. Most of these toxicants attack and impair the metabolic machinery and disturb the natural redox state in the fish leading to a condition of oxidative stress. In oxidative imbalance, there is an increased production of ROS which ultimately leads to cell death resulting in organ failure (Details in chapter 2, Section 2.6). MCs exert their toxicity by inhibition of a class of enzymes called Phosphatases. Protein phosphatases are a group of enzymes, found ubiquitously, which

are responsible for the dephosphorylation of various proteins and enzymes in a cell. Their role is an extremely important one since protein phosphorylation and dephosphorylation are required for the regulation of a large number of cellular and metabolic activities (Details in Chapter 2, section 2.3). Inhibition of these enzymes can lead to the perturbation in the metabolic pathways which could ultimately lead to fatal consequences. Since the biochemical pathways in the organ systems do not function in isolation, inhibitory action on one set of enzymes in a particular metabolic pathway can have deleterious effects on other pathways as well.

Earlier studies done on acute exposure of MCs (oral routes, mainly restricted to intracellular MCs) in fish have shown excessive liver damage resulting in fatal consequences (Refer to Chapter 2, section 2.3, 2.5). However, chronic exposure to extracellular MCs may not result in fatality, but it could still result in impairment of the biochemical processes affecting the functioning of organisms. To understand the impact on the cellular processes, it is important to study the metabolic pathways as they are a series of chemical reactions occurring in the cell. Any adverse effects on the cell would thus be reflected as changes occurring at metabolic level. Studying the metabolic pathways would not only aid in a better understanding of toxicity mechanisms, but also lead to the identification of potential biomarkers that can be used for risk management when HABs occur in natural waters.

The science of a comprehensive analyses of small molecules, the metabolites, in a biological system is called Metabolomics. Metabolome of any living organism comprises metabolites, which are the intermediates and products of metabolism. Metabolomics is

often known as systematic investigation of the unique chemical fingerprints that specific cellular processes leave behind (Daviss, 2005). Metabolomics use techniques from analytical chemistry aiming to measure as many metabolites as possible linking the resulting data to biochemistry, biology, and physiology. Metabolites are characterized by a diverse chemistry and therefore require the application of numerous analytical approaches for their extraction, separation, detection, and quantification. In the past decade, the analytical techniques have improved substantially, allowing the analyses of thousands of metabolites in different kinds of sample matrices of plant and animal origin simultaneously (Griffin and Bollard, 2004). There are a number of high-throughput analytical instruments available such as LC/MS (Liquid chromatography-mass spectrometry), GC-MS (Gas chromatography- mass spectrometry) which are used for the analyses of metabolites. Metabolomics has been applied in various research areas including environmental and biological-stress studies (Rosenblum et al., 2005), biomarker discovery (Griffiths et al., 2010), functional genomics (Saito et al., 2010) and integrative systems biology (Oliver et al., 1998). Metabolomics is considered by many to be complementary to genomics, transcriptomics, and proteomics (Griffin and Bollard, 2004 and Lindon et al., 2004).

In environmentally related studies, metabolomics is of great importance as it analyzes the last step in the series of changes that occur following a toxic exposure. Targeting the metabolic machinery could provide valuable insights into the mechanisms of toxicity and other toxicological targets of environmental pollutants such as MCs. Analyses of metabolites often presents with a lot of details about the functioning of cellular processes that are of utmost importance to the organism in concern. Following a toxic exposure,

the key cellular processes would be disrupted which could be easily identified through the systematic study of metabolites. Since MCs inhibit protein phosphatases which are important for regulation of key cellular processes, studying the metabolites upon exposure to MCs could provide deep knowledge regarding the toxicological targets and underlying toxicity mechanisms. Chronic exposure to extracellular MCs would not only impair the cellular processes due to irreversible inhibition of phosphatases, but also have other possible implications on the biological system as a whole.

However, there is no report available to-date on metabolite profiling following exposure of aquatic organisms such as fish to MCs. This is the first attempt to understand the effects of extracellular MCLR and MCRR on the metabolism of zebrafish organs. It is aimed at understanding the toxicological mechanism of action of MCLR and MCRR in balneation route.

7.2 Methodology

7.2.1 Fish exposure and sampling

Chemicals, reagents used for these experiments are given elsewhere in Chapter 3 (section 3.2.1, 3.2.2). Details about selection and procurement of zebrafish are given in Chapter 3 (section 3.4.1 and 3.4.2).

Zebrafish were exposed to MCLR and MCRR dissolved in aquarium water at concentrations of 10 µg L^{-1} for a period of 30 days. The concentration of MCLR and MCRR for the exposure was decided based on the previous experiments conducted in this study namely, the biochemical changes upon a balneation exposure (dose-response study) and the analytical measurements done for detection of MCLR and MCRR in real water

samples (6.2-9.2 µg L^{-1}). Upon exposure of zebrafish to MCLR and MCRR at 10 µg L^{-1}, it was found that the biochemical enzyme activities were reduced significantly in the zebrafish organs (Chapter 4 and 5). Hence, 10 µg L^{-1} of MCLR/MCRR was used to conduct these following experiments. Briefly, stock solutions of MCLR and MCRR were prepared (10 mg L^{-1}) and were diluted to obtain the required concentrations. Water in the fish tanks was replaced with fresh water spiked with the toxins on a daily basis. Dosing concentrations for MCLR and MCRR (10 µg L^{-1}) were confirmed by measurements using liquid chromatograph, composed of an HP100 liquid chromatograph (Agilent Technologies, U.S.A) interfaced with a triple quadrupole MS/MS (Applied Biosystems, U.S.A). Analytical separation was achieved on a Zorbax Extend-C18 5 µm, 2.1 x 150 mm (Agilent technologies, Germany). The injection volume was 10 µL. The mobile phase consisted of 0.1 % formic acid, HCHO (solvent A) and methanol, CH_3OH (solvent B). A gradient elution was used, starting with water: methanol at 90:10 from 0 to 6 min, and switching to 5:95 up to 10 min before returning to the original conditions to re-equilibrate the system (as done for biochemical experiments, chapter 5).

The capillary voltage was set at 89 volts and the cone voltage at 4 volts. The desolvation gas (nitrogen) temperature and the gas flow-rate were set at 350°C and 615 L/h, respectively. The ion source temperature was set at 120°C. LC-MS-MS was operated in the positive ion mode. MCLR and MCRR were monitored by using the MS instrument in the SRM mode: m/z 995.6; fragment ion at 135.1 and m/z 520; fragment ion 135.1, respectively.

At the end of the exposure period, zebrafish (n= 5 per group) were collected and the organs (gills, liver, intestine and brain) were harvested, snap frozen and then lyophilized Figure 3.4, Chapter 3).

7.2.2 Extraction of metabolites

Following the lyophilization, tissues were homogenized and sequentially extracted with methanol, water and chloroform. At the end of the extraction procedure, polar and non polar extracts from each tissue were obtained. The polar extract mainly constituted polar metabolites from the respective tissues, while the non-polar extract contained metabolites from lipid classes and other families which are immiscible with water. The extracts were then dried using speedvac (Details in section 3.5.4, Chapter 3), and were stored in the dry box at ambient temperatures until analysis.

7.2.3 Polar and non polar metabolite analyses

The dried extracts were reconstituted using specific solvents (50 µL of 50% methanol for polar extracts and 100 µL of 50:50 methanol: butanol containing 10 mM ammonium formate) and then injected into different analytical platforms as given below to obtain the metabolic fingerprint (Details in Chapter 3, section 3.5.4). Polar extracts were analyzed by RP-LC-MS (Reverse phase- Liquid chromatography- mass spectrometry) in positive mode and RP-LC-MS in negative mode in an untargeted or "profiling" manner using the instrumentation and methods as described elsewhere in Chapter 3, section 3.5.4.

The non-polar extracts were analyzed by RP-LC-q-TOF (Reversed phase-liquid chromatography-quadrupole time of flight mass analyzer) in the positive mode and in the

negative mode (lipidomics methods) in an untargeted or “profiling” manner using the instrumentation and methods described elsewhere in Chapter 3, section 3.5.4.

The analyses of metabolites were done using five biological replicates.

7.2.4 Data mining

Following the instrumental analyses, analytical data were extracted using MZ mine. The raw data file obtained from the chromatographic system was first imported to MZ mine. The raw data after import were then filtered. Filtering was done using crop filters; Scan filters (filters the data scan by scan); Mean filters (for each data point, the filter assigns to it the intensity average of all the data points inside the user defined window, which is centered in the mass value of this data point); m/z resample filter (each scan is divided in m/z bins whose length is defined by the user in the parameters. The mass of the new data point is in the middle of each m/z bin's space). After filtering, the files containing the filtered data were used for baseline correction. It compensates for gradual shifts in the chromatographic baseline by detecting the baseline and then subtracting it from the raw data intensity values, following which, peak detection was done. Peak detection was performed in three steps by the software. First, the individual ions were detected in each mass spectra. This step is called “Mass detection”. A mass list was generated for each mass spectrum in this step. The second step was chromatogram construction, where the ions found in consecutive scans were connected together to form chromatograms. The last step was deconvolution, where chromatograms were separated into individual peaks. After Peak detection, peak extension module was used to extend the peaks in a peak list in both directions of the retention time. The purpose was to mainly add missing data to

peak lists produced Peak List Builder in the previous step. This was followed by peak modeller module which was used to fit the data points of a peak with a given model. Later, Peak alignment and gap filling was done. The former was done to match corresponding peaks in the peak lists of multiple samples while the latter assisted in filling the gaps in the peak list when it is possible according to the defined parameters.

At this stage, the file containing the peak list with the relevant information was exported in csv format for further statistical and visualization procedures using the R software. The imported peak lists were first normalized and then tested for significance using R scripts on the R software interface. One-way ANOVA was performed with multiple comparisons as a post hoc test on all the samples to assess the significance. p-values obtained from the multiple comparisons were further adjusted using False discovery rate adjustments (FDR), following which, the significant metabolites (m/z) were analyzed for fold changes. After obtaining the data sets (from m/z mine) containing metabolites from zebrafish tissues, data were log normalized using an 'R script'. Normalized data were then statistically analyzed (ANOVA with multiple comparisons, $p<0.05$; p value correction). The m/z ratios that were statistically significant in zebrafish tissues exposed to MCLR and MCRR as compared to controls were then evaluated for their fold changes. A final list of m/z ratios was then obtained satisfying both the conditions ($p<0.05$, > 2-fold change) for different experimental pairs (MCLR vs. Control; MCRR vs. Control and MCLR vs. MCRR). After obtaining the significant m/z ratios among different groups in different conditions, the list of masses were analyzed for metabolite identities and biochemical pathway mapping (i.e. identified metabolites from the significant masses were mapped on biochemical pathways occurring in zebrafish to understand the situation

at biochemical level). For identification and pathway mapping, METDET software was used. The software provided tentative identities of the m/z based on their neutral masses. After obtaining the tentative identities, the metabolites were mapped on biochemical pathway mapping module in the software. Mapping is useful to understand the pathways which are disturbed or perturbed following the exposure to MCLR/MCRR in zebrafish organs.

7.3 Results and Discussion

Following the analyses of polar and non polar metabolites in zebrafish organs after a balneation exposure to MCLR and MCRR (10 µg L^{-1}) it was found that a number of metabolites were significantly perturbed in various organs of zebrafish under different experimental groups (see supplementary sections, tables S 1 to S 4). These metabolites belonged to various biochemical pathways involving cell regulation, signaling and metabolism of macromolecules such as carbohydrates, lipids and proteins in the zebrafish organs. It shows that all these pathways were affected following an exposure to MCLR/MCRR in the balneation route. A total of 32 metabolic pathways were affected in various organs following the exposure to MCLR and MCRR (Table 7.1). Out of these pathways, 7 biochemical pathways belonged to carbohydrates; 6 pathways to proteins and 11 pathways to lipid metabolisms. The remaining 8 biochemical pathways belonged to hormone synthesis, drug metabolism, nucleotide metabolism and histamine receptors.

Table 7.1

Biochemical pathways affected in zebrafish organs following a balneation exposure to MCLR/MCRR.

Lipid Metabolism	Protein Metabolism	Carbohydrate Metabolism	Others
Fatty acid oxidation	Valine, leucine, isoleucine degradation	Glycolysis/gluconeogenesis	Steroid biosynthesis
Glycerophospholipid metabolism	Tyrosine metabolism	Pentose and glucuronate interconversions	Steroid hormone biosynthesis
Fatty acid elongation	Lysine metabolism	Peptidoglycan biosynthesis	glucocorticoid synthesis
Linoleic acid metabolism	Phenylalanine metabolism	Starch and sucrose metabolism	Drug metabolism cytochrome P450
Bile acid synthesis	Tryptophan metabolism	Pentose Phosphate pathway	Histamine receptor agonists
Biosynthesis of unsaturated fatty acid	Selenoamino acid metabolism	Citric Acid cycle (TCA) Amino sugar and nucleotide sugar metabolism	Glutathione metabolism
Arachidonic acid metabolism			Purine metabolism
α – linolenic acid metabolism			Pyrimidine metabolism
Secondary bile acid synthesis			
Prostaglandins			
Eiconosoid metabolism			

7.3.1 Lipid Metabolism

It was found that 11 biochemical pathways were affected in lipid metabolism (Table 7.2). Out of which, 3 pathways belonged to a class of essential fatty acids (Linoleic acid metabolism, α – linoleic acid metabolism and Arachidonic acid metabolism). Linoleic (ω-6) and α – linolenic fatty acids (ω-3) are essential fatty acids which are required for the functioning of other biological processes apart from acting as fuel (Goodhart and Shils, 1980). Arachidonic acid is a polyunsaturated fatty acid present in the phospholipids, especially phosphatidylethanolamine, phosphatidylcholine, and phosphatidylinositides) of membranes of the body's cells, and is abundant in the brain, muscles, liver (Baynes and Marek, 2005). In addition to being involved in cellular signaling as a lipid second messenger involved in the regulation of signaling enzymes, such as PLC-γ, PLC-δ, and PKC-α, -β, and -γ isoforms, arachidonic acid is a key inflammatory intermediate (Baynes and Marek, 2005). Arachidonic acid also serves as a precursor in the production of eicosanoids (discussed later in this section)

Table 7.2

Specific pathways affected in lipid metabolism in various zebrafish organs following a balneation exposure to MCLR and MCRR

Lipid Metabolism	Organs	Experimental Group	Occurrence
Fatty Acid oxidation	Gills	LR/C; LR/RR	2
Glycerophospholipid metabolism	Gills	LR/RR	2
Fatty acid elongation	Gills	LR/RR	1
Linoleic acid metabolism	Gills	LR/RR	3
	Intestine	LR/C	
	Brain	LR/RR	
Bile acid synthesis	Intestine	RR/C	2
	Liver	RR/C	
Biosynthesis of unsaturated fatty acid	Intestine	LR/RR	1
Arachidonic acid metabolism	Gills	LR/C ; LR/RR	5
	Brain	RR/C; LR/RR	
	Intestine	LR/RR	
α – linoleic acid metabolism	Brain	LR/RR	2
	Liver	LR/RR	
Secondary bile synthesis	Liver	RR/C	1
Prostaglandins	Intestine	LR/RR	2
Eiconosoid metabolism	Intestine	LR/RR	1

Note: LR/C – MCLR exposed vs. Control group; RR/C – MCRR exposed vs. Control group; LR/RR – MCLR vs. MCRR group

It is well established that fish require these long-chain highly unsaturated fatty acids (HUFA) for normal growth and development, including reproduction (Sargent et al., 1999). As mentioned, these fatty acids play important physiological roles in fish as components of membrane phospholipids and as precursors of biologically active eicosanoids (Sargent et al., 1995; Sargent et al., 2002). Arachidonic acid is the major eicosanoid precursor in fish cells (Bell et al., 1994) and thus these essential fatty acids play a key role in maintaining the overall health of the fish (Mustafa and Srivastava, 1989; Sorbera et al., 1998). These observations about the functional role of these essential fatty acids suggest that any disturbance in the metabolism of these essential and important fatty acids could affect a vast number of cellular and biological processes. Prostaglandin synthesis and eicosanoid metabolism were also affected upon exposure to MCLR and MCRR in the current study which could be due to the disruption of essential fatty acid synthesis as described above since these fatty acids are precursor molecules for prostaglandin synthesis. Lister and Kraak, (2008) have demonstrated the role of prostaglandins in zebrafish reproduction. Prostaglandins, the subclass of eicosanoids, have been shown to play essential roles in the development of fish (Cha et al., 2006). There are four families of eicosanoids—the prostaglandins, prostacyclins, the thromboxanes and the leukotrienes. For each, there are two or three separate series, derived either from an ω-3 or ω-6 essential fatty acid. Apart from their role in reproduction, there are early reports in the literature, which suggested that eicosanoids might influence the immune system of fish (Laudan et al., 1986). Their experiments suggested the role of prostaglandins in the immunosuppressive behavior (Laudan et al., 1986). These results obtained suggests that exposure to MCLR and MCRR could

possibly affect a lot of interlinked lipid metabolic pathways in zebrafish, which could affect reproduction (Figure 7.1). Reproductive behavior, if affected, could lead to disturbance at ecological levels which makes it an area of serious concern.

Apart from affecting essential fatty acids and their related compounds, exposure to MCLR and MCRR also resulted in perturbation of primary and secondary bile acid synthesis (Table 7.2). However, the effect on bile acid synthesis was seen primarily for fish exposed to MCRR. Fish bile is produced in the liver, stored in the gall bladder and delivered to the intestine or pyloric caeca via the bile duct (Henderson and Tocher 1987). Dietary lipids are hydrolyzed by lipases and emulsified by bile acids in the gut, from where they are absorbed (Henderson and Tocher 1987).

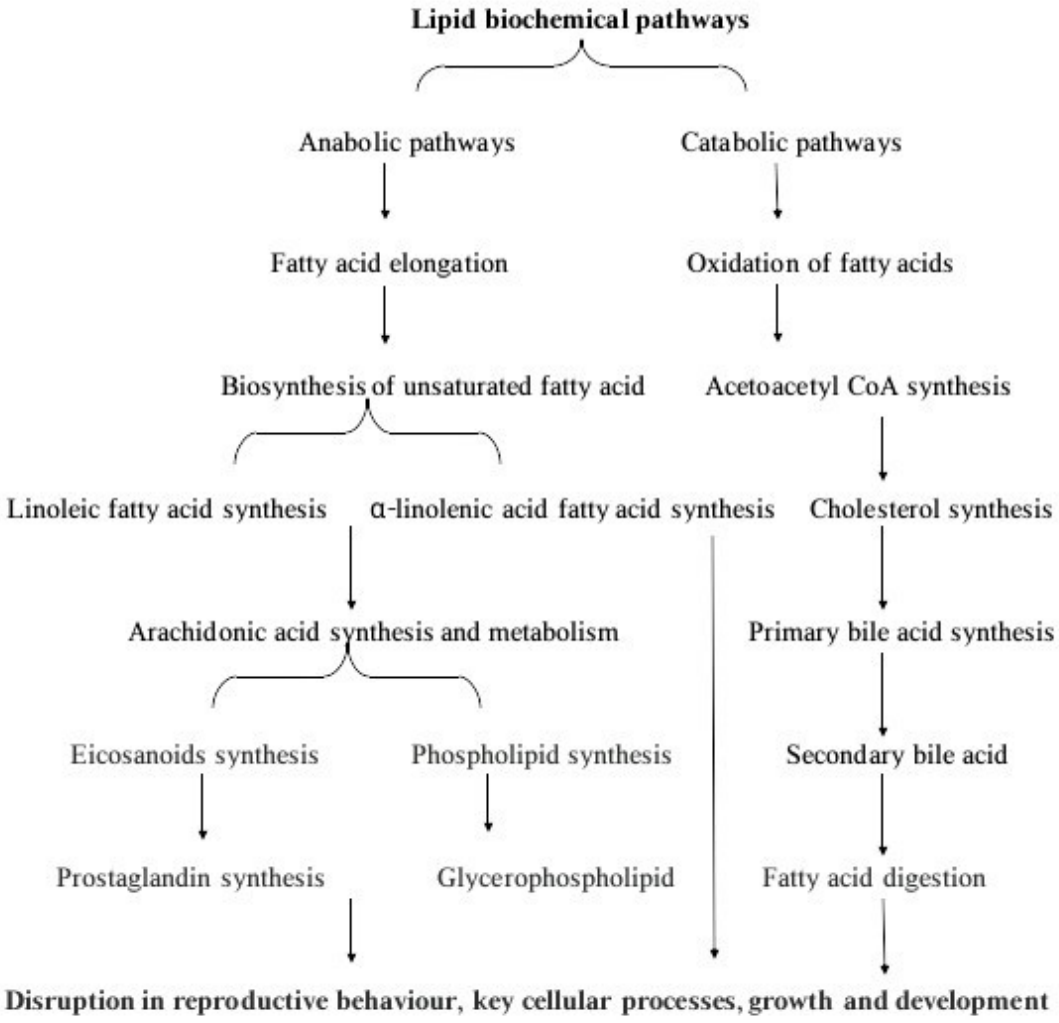


Figure 7.1: Lipid maps- Interlinkage between different lipid biochemical pathways identified by Kegg in the present study.

If the synthesis of bile acids is impaired, it would cause disturbance in the entire lipid metabolism as a whole (Smith et al., 2012). In the current set of experiments, lipid oxidation, fatty acid elongation and synthesis of unsaturated fatty acids are found to be perturbed in different organs as shown in Figure 7.2-7.3. Apart from regular lipids, special lipids (phospholipids) were also observed to be affected by the balneation

exposure. A member of phospholipids family, glycerophospholipid was affected in the case of fish exposed to MCRR. These lipids are components of biological membranes. They are essential for the integrity of the cell membranes. If the pathways concerning these phospholipids are altered, it would result in altered and impaired cell functions (Figure 7.1).

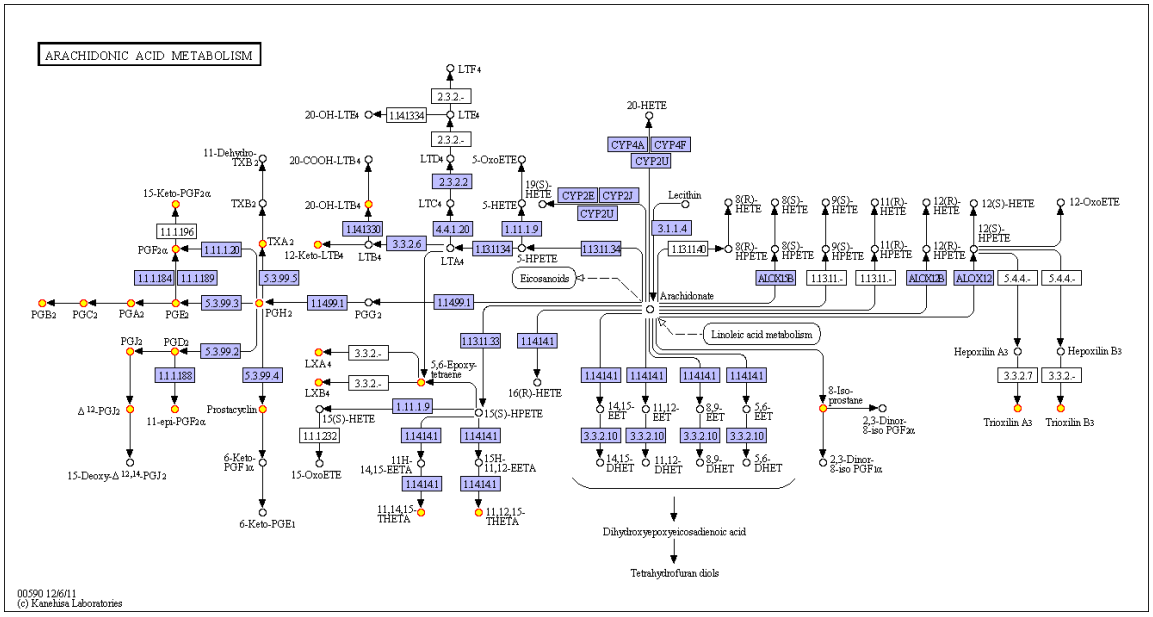


Figure 7.2: Arachidonic acid metabolism: Metabolites indicated as yellow solid circles were perturbed following an exposure to MCLR/MCRR in zebrafish gills.

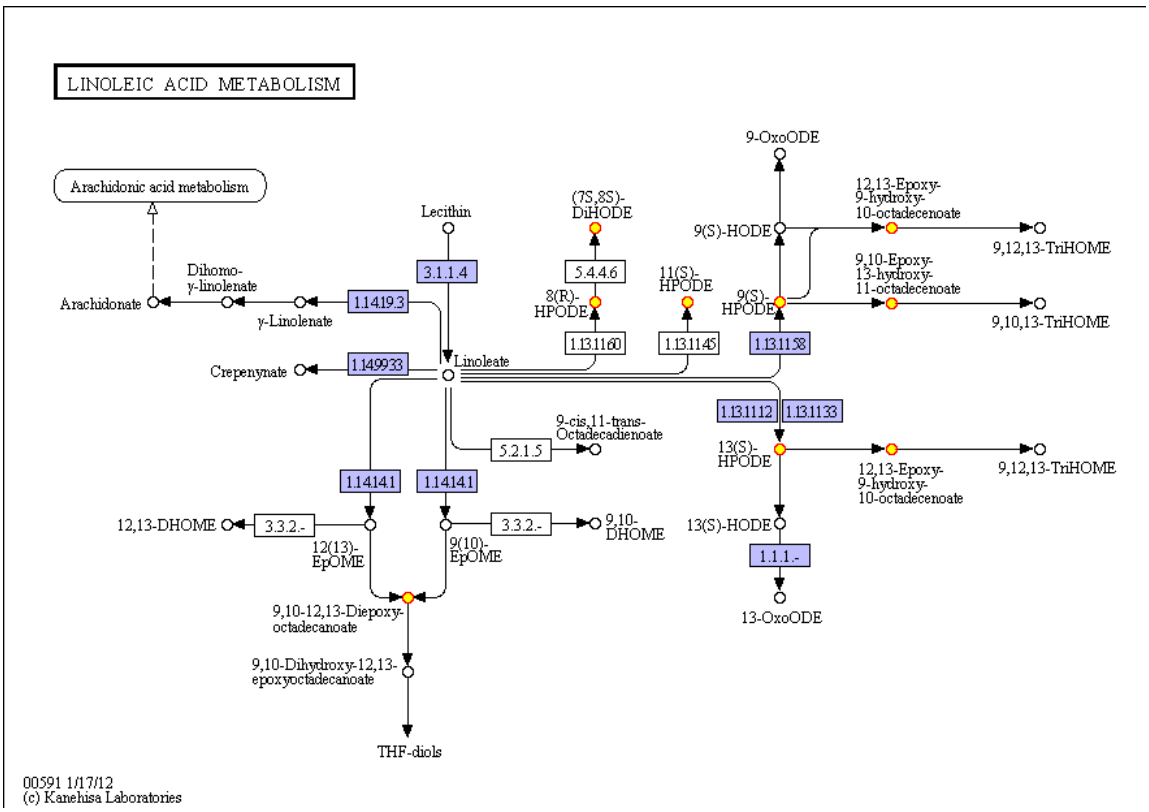


Figure 7.3: Linoleic acid metabolism: Metabolites indicated as yellow solid circles were perturbed following an exposure to MCLR/MCRR in zebrafish intestine

In general, biochemical pathways involving lipids were altered in all the organs exposed to MCLR and MCRR in the present study (Table 7.2, Figure 7.1). It can be observed that all the biochemical pathways belonging to lipid classes are interlinked; perturbation in one has led to the disruption of other pathways as well in the cascade. Continual exposure can thus lead to deleterious effects not only on the exposed organisms, but also on the entire ecosystem as a whole (Figure 7.1).

Among all the organs studied in the present experiment, gills were the most affected followed by liver and intestine. The least affected organ was brain. Since the exposure route was through gills, the residence time of MCLR/MCRR in this tissue would be the longest, which could be the possible reason for disruption of a number of pathways in

gills as shown in Table 7.2. Intestine and liver are major organs for lipid metabolism since they are the sites where digestion and subsequent absorption of lipids take place so these organs are bound to be affected. Brain was observed to be affected only for essential fatty acid metabolism which is justified since brain contains high levels of these fatty acids for different processes and its proper functioning. The complete list of metabolites identified in each organ belonging to lipid classes is given in the Supplementary section (Table S 1 to S 4). All the lipid class metabolites were identified in non polar extracts of organs belonging to different experimental groups.

7.3.2 Protein Metabolism

It was found that 6 biochemical pathways were affected in protein/amino acid metabolism (Table 7.3), out of these pathways, 5 belonged to the class of essential amino acids, namely, valine/leucine, tyrosine, lysine, phenylalanine and tryptophan (Ketola, 1982). Amino acids are the building blocks of all proteins. Amino acids are required by the fish not only for their normal growth and development, but also for regulation of key metabolic pathways involved in reproduction, immune response, osmoregulation, cell signaling, antioxidant and stress response (Li et al., 2009). Each of these above mentioned amino acids attacked by MCLR/MCRR in various tissues has a distinct role to play in proper growth and development of the organisms. Lysine levels critically affect fish growth performance and health (Mai et al., 2006). Lysine is a substrate for the synthesis of carnitine, which is required for the transport of long chain fatty acids from the cytosol into mitochondria for oxidation. Carnitine also protects against the toxicity of ammonia and xenobiotics, improves acclimation to extreme temperature changes and

associated stress, and enhances reproduction performance (Harpaz 2005). Since lysine synthesis is affected following an exposure to MCLR and MCRR, there is a highly likelihood that carnitine levels would be affected as well since lysine is the precursor for carnitine (Harpaz, 2005). Low levels of carnitine could thus be responsible for adverse effects on health and growth performance of zebrafish.

Disruption in lysine metabolism can derange the fatty acid oxidation process along with other possible implications. The fatty acid oxidation pathway was also perturbed as a result of MCLR/MCRR exposure as discussed in the previous section. Disruption of fatty acid oxidation and lysine metabolism clearly indicates theinterlink between different metabolic pathways which is disrupted in the organ systems by MCLR/MCRR upon balneation exposure.

Table 7.3

Specific pathways affected in lipid metabolism in various zebrafish organs following a balneation exposure to MCLR and MCRR

Protein Metabolism	Organs	Experimental Group	Occurrence
Valine, leucine, isoleucine degradation	Gills	RR/C; LR/RR	4
Tyrosine metabolism	Gills Intestine	LR/C; LR/RR LR/C	3
Lysine biosynthesis	Gills	LR/RR	1
Phenylalanine metabolism	Gills Intestine	LR/RR LR/C	2
Tryptophan metabolism	Intestine	LR/RR	2
Selenoamino acid metabolism	Brain	LR/RR	1

Note: LR/C – MCLR exposed vs. Control group; RR/C – MCRR exposed vs. Control group; LR/RR – MCLR vs. MCRR group

Phenylalanine is also important for key cellular processes. Phenylalanine can be converted to tyrosine by the liver and kidneys of aquatic organisms. Hence, disruption in one amino acid metabolism would largely affect the other amino acids as well. In the present study, metabolisms for both tyrosine and phenylalanine were disrupted. Tyrosine is a common precursor for important hormones and neurotransmitters, including thyroxine (T4), triiodothyronine, epinephrine, norepinephrine, dopamine, and melanin. These molecules have important regulatory roles (Chang et al., 2007; Yoo et al., 2000); hence, dietary levels of phenylalanine and tyrosine could profoundly influence pigmentation development, feed intake, growth performance, immunity, and survival of fish in natural environments.

Leucine and isoluecine are known to be involved in immunity modulation and cell signaling in fish (Figure 7.4) (Li and Gatlin 2007). Tryptophan is known for its involvement in neurotransmitter and stress responses, and can be converted to serotonin (5-hydroxytryptamine; a neurotransmitter) and melatonin (hormone; an antioxidant) (Fang et al. 2002). Serotonin is involved in modulation of cortisol release, behavior and feeding while melatonin is known to improve testicular development. Thus, disruption of tryptophan metabolism could also indirectly affect the reproduction. Adverse effects on reproduction could lead to a massive imbalance in the ecosystem as discussed in the previous section. Effects on reproductive health could completely alter the aquatic community affecting all the levels in the hierarchy.

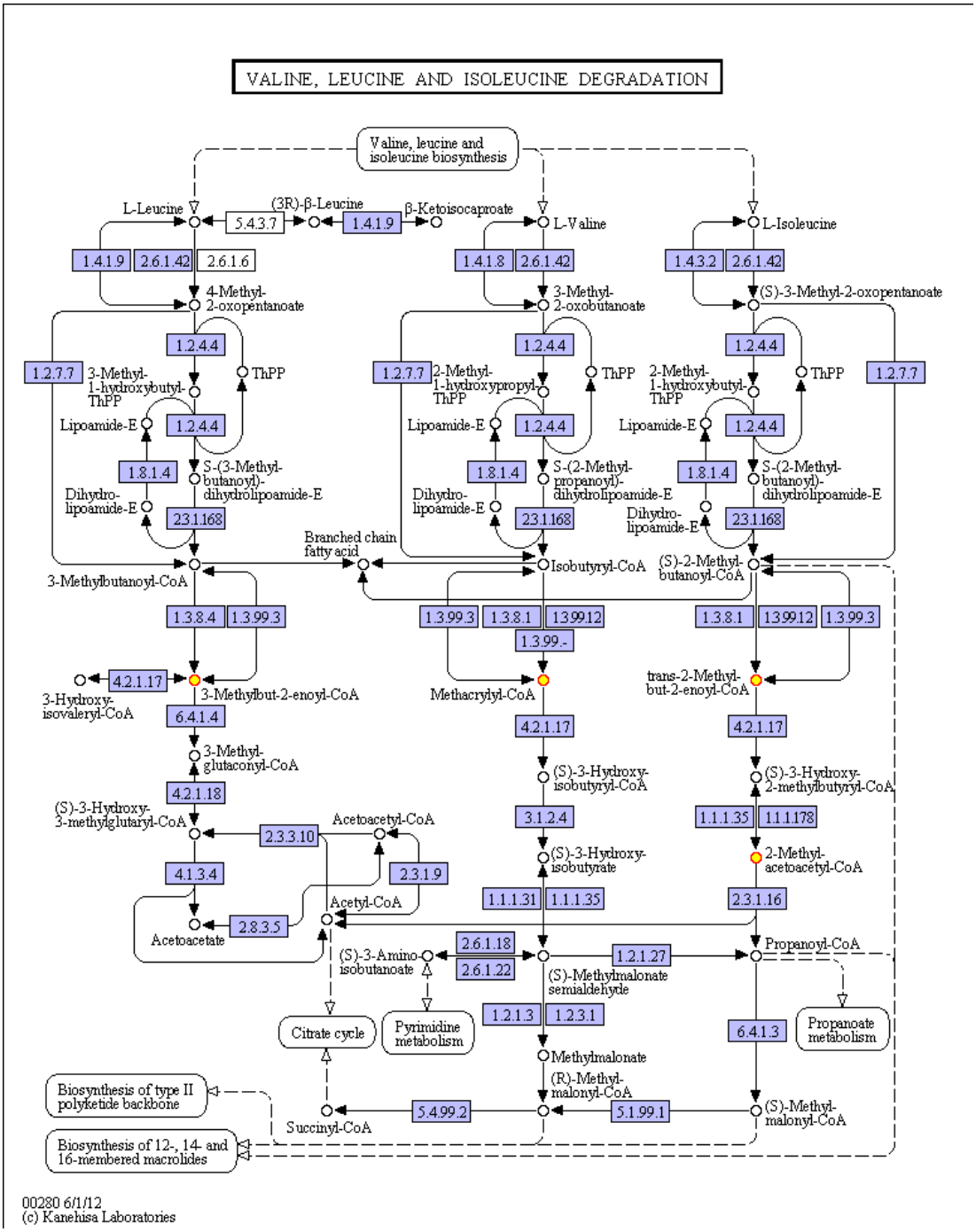


Figure 7.4: Valine/Leucine/Isoleucine degradation: Metabolites indicated as yellow solid circles were perturbed following an exposure to MCLR/MCRR in zebrafish intestines

Apart from the essential amino acids discussed in the previous section, there is a special class of amino acids known as selenoamino acids. In the latter amino acids, selenium (Se) has been substituted for sulfur. Selenoamino acids include selenocysteine, selenohomocysteine and selenomethionine (Figure 7.5). These amino acids can be incorporated into body proteins.

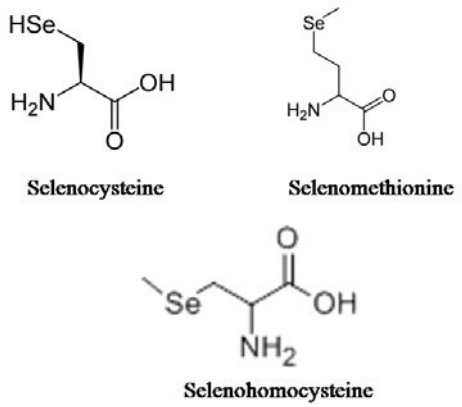


Figure 7.5: Structure of selenoaminoacids

This allows Se to be stored in the organism and reversibly released by normal metabolic processes. Selenium is necessary for proper formation and functioning of GPx, which is a major cellular antioxidant enzyme (as discussed elsewhere in Chapter 5). This enzyme protects cell membranes from damage or lysis due to lipid peroxidation. Without adequate selenium, normal cellular and organ metabolism break down because of peroxides produced as a by-product of digestion (Dennis 1998). It is evident from the present study that selenoamino acids metabolism could indirectly affect GPx levels as well. This relation between the selenoamino acids and GPx activities could possibly serve as a link to the previous observations reported in Chapter 5 wherein, it was

observed that levels of GPx and other glutathione based enzymes decreased in some organs following an exposure to MCLR/MCRR in balneation route. In general, biochemical pathways involving amino acids/proteins were altered mainly in gills and intestine tissues exposed to MCLR and MCRR (Table 7.3).

Out of the 6 amino acid pathways which were perturbed following a balneation exposure with MCLR/MCRR zebrafish organs, metabolites from gills resulted in perturbation of 4 amino acid pathways followed by intestine metabolites which resulted in disruption of 3 amino acid metabolic pathways. Brain showed perturbation in only selenoamino acid metabolism. Since these amino acids are a major source of Se, which is an essential metal for antioxidant formation, disruption in seleamino acid metabolism could be of serious concern. Brain contains a lot of unsaturated and essential fatty acids which require antioxidants for their stabilization. It is very likely that implications from amino acid metabolism could also thus impair lipid and carbohydrate metabolism.

The complete list of metabolites identified in each organ belonging to amino acid class is given in the Supplementary section (Table S 1 to S 4). All the amino acid class metabolites were mainly identified in polar extracts of organs belonging to different experimental groups.

7.3.3 Carbohydrate Metabolism

It was found that 6 biochemical pathways were affected in carbohydrate metabolism (Table 7.4), out of which 3 belonged to glucose metabolism, namely, glycolysis/gluconeogenesis, citric acid cycle and pentose phosphate pathways.

Table 7.4

Specific pathways affected in lipid metabolism in various zebrafish organs following a balneation exposure to MCLR and MCRR

Carbohydrate metabolism	Organs	Experimental Group	Occurrence
Glycolysis/gluconeogenesis	Brain	LR/RR	1
Pentose and glucuronate interconversions	Gills	LR/C	4
	Liver	LR/RR	
Sucrose metabolism	Gills	LR/C	1
Pentose Phosphate pathway	Intestine	LR/RR	1
Citric Acid cycle (TCA)	Brain	LR/RR	1
Amino sugar and nucleotide sugar metabolism	Brain	LR/RR	1

Note: LR/C – MCLR exposed vs. Control group; RR/C – MCRR exposed vs. Control group; LR/RR – MCLR vs. MCRR group

MCs are known to inhibit protein phosphatases. Phosphatases are enzymes that act by removing a phosphate moiety from the proteins. Removal of phosphate group of specific intracellular proteins is believed to be an important and versatile mechanism for regulating their biological activity, which, in turn, controls a variety of cellular functions (Details given in Chapter 2, section 2.3). Phosphatases play a very important role in glycogen metabolism. Glycogen is a polysaccharide, and its building block is glucose. Excess glucose is channeled to glycogen sythesis by glycogen synthase enzyme for its conversion to glycogen. Glycogen synthase enzyme is regulated by protein phosphatase which is known to be inhibited by MCs (Saltiel, 2000). A number of studies reported in the literature have shown that there is a marked depletion in glycogen upon

administration of MCs in various organisms (Guzman and Solter, 2002; Gehringer et al., 2004). Since glycogen metabolism is regulated by glucose levels in the cells, any adverse effects to glycogen metabolism would tend to have implications on the entire glucose metabolism. It is clear from the results obtained in this study that inhibition of phosphatases affected all the metabolic pathways of glucose as shown in interlinked pathway map in Figure 7.6.

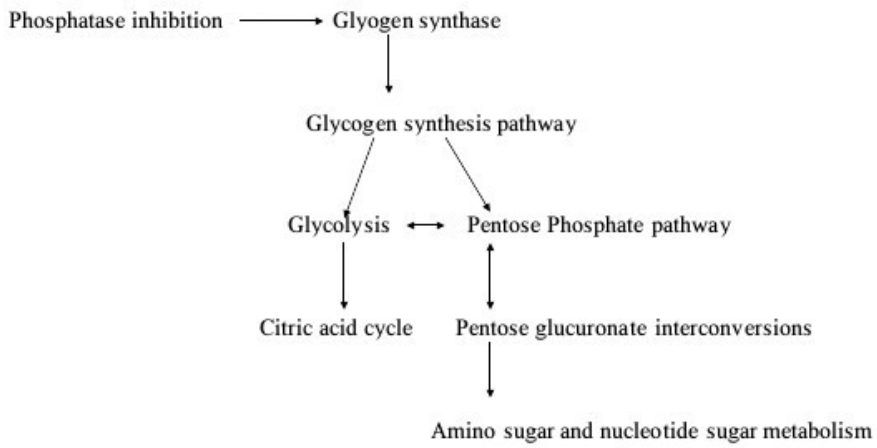


Figure 7.6: Inhibition of protein phosphatases leading to adverse effects on other carbohydrate metabolic pathways

Results also show perturbation in the amino and nucleotide sugar metabolism (Table 7.4). Nucleotide sugars are the activated forms of monosaccharides. Nucleotide sugars act as glycosyl donors in glycosylation reactions. Those reactions are catalyzed by a group of enzymes, called glycosyltransferases. These moieties also play important role in many cellular processes and a small disruption in their metabolism could affect many metabolic pathways. In the present experiment, we found this nucleotide metabolism to be deranged in brain tissue following the exposure to MCs. Apart from these pathways,

sucrose metabolism and pentose/glucuronate interconversion pathways were also affected on MCLR/MCRR exposure to organs of zebrafish (Figure 7.7). However, the significance of these metabolic pathways to any biological functions in fish and aquatic organisms is not available in the literature.

Out of the 6 biochemical pathways, metabolites from brain resulted in perturbation of 3 carbohydrate metabolism pathways followed by gills metabolites which resulted in disruption of 2 carbohydrate metabolic pathways. Brain showed perturbation in glucose metabolism pathways (Figure 7.8). Glucose is the only sugar utilized by the brain. Any disruption in the glucose availability, or excess could lead to deleterious effects to the brain cells causing cell death. Since brain is the control centre for the entire body of the organism, any serious impairment in brain tissue is of considerable concern. Interestingly, most the metabolites belonging to these pathways were found significantly different among MCLR and MCRR exposed tissues. Close observations of the fold changes show that tissues exposed to MCLR have higher changes in these metabolites (characterized by higher fold changes), as compared to that in MCRR. This difference is conceivable since MCLR is more toxic and lethal than MCRR.

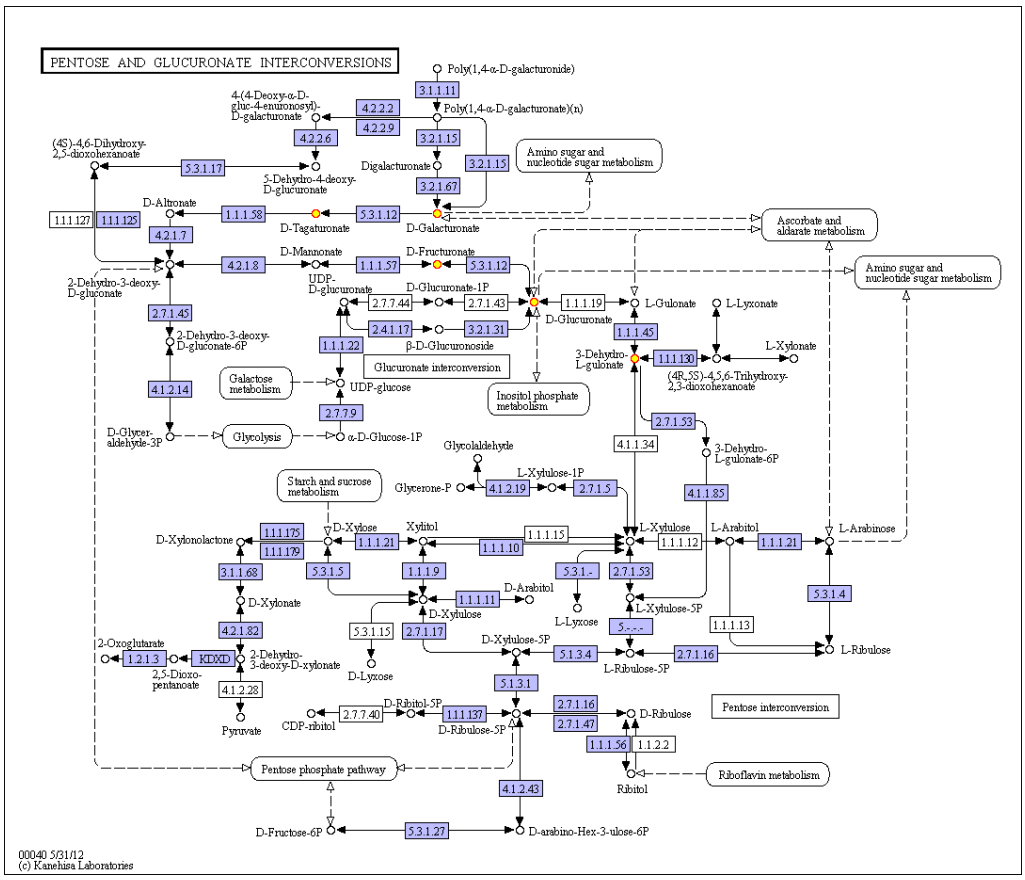


Figure 7.7: Pentose and glucoronate interconversions: Metabolites indicated as yellow solid circles were perturbed following an exposure to MCLR/MCRR in zebrafish gills

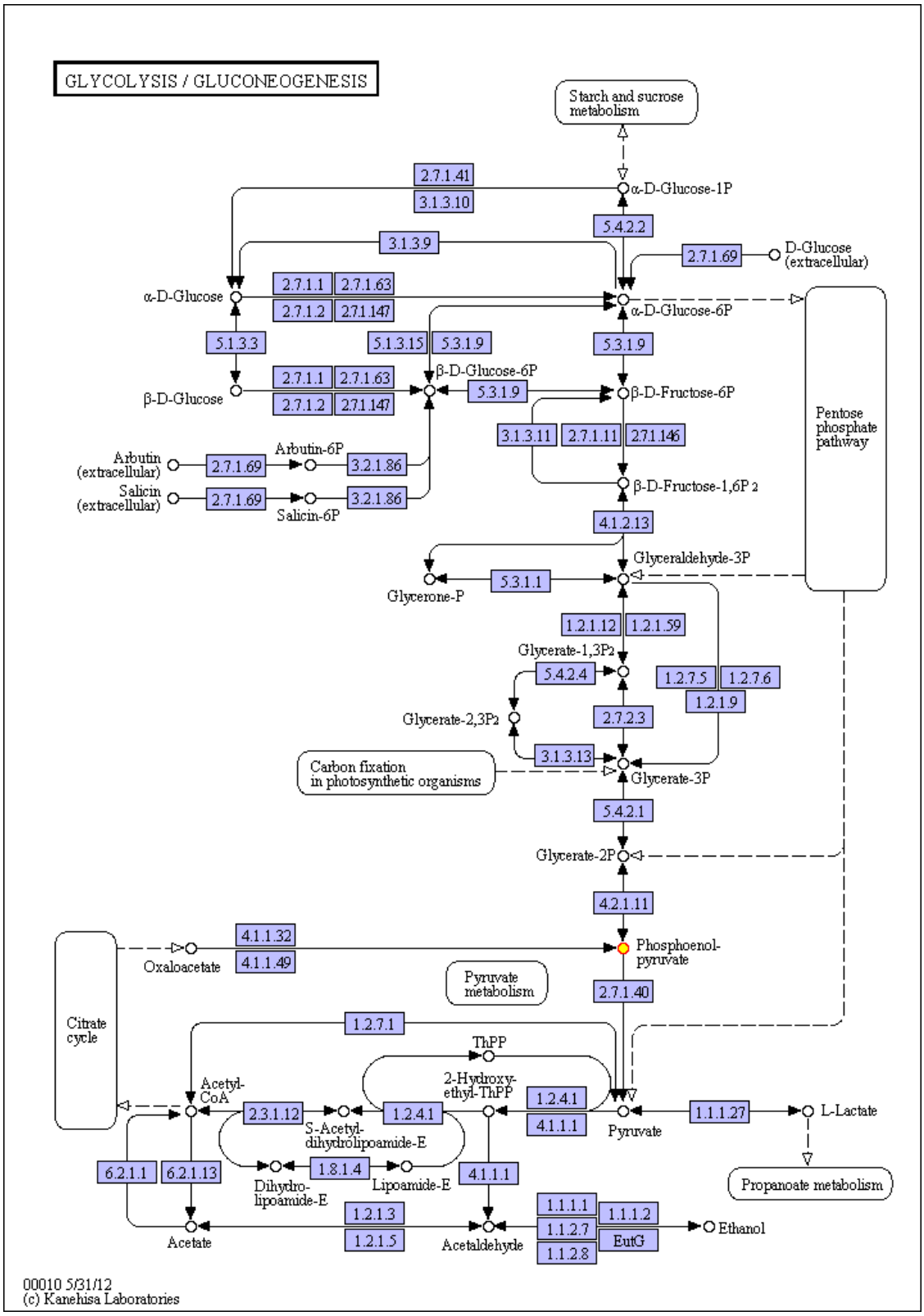


Figure 7.8: Glysolysis: Metabolites indicated as yellow solid circles were perturbed following an exposure to MCLR/MCRR in zebrafish brain

The complete list of metabolites identified in each organ belonging to amino acid class is given in the Supplementary section (Table S 1 to S 4). All the carbohydrate class metabolites were mainly identified in polar extracts of organs belonging to different experimental groups.

7.3.4 Other metabolic pathways

A total of 8 other biochemical pathways got disturbed following the exposure to MCLR/MCRR (Table 7.5). These metabolic pathways do not belong to class of macromolecules. Among them, 3 biochemical pathways belong to a class of steroid hormones (lipid and phospholipid derived hormones) and its metabolites, namely, steroid synthesis, steroid hormone biosynthesis and glucocorticoid synthesis (Figure 7.9).

Table 7.5

Specific pathways affected in lipid metabolism in various zebrafish organs following a balneation exposure to MCLR and MCRR

Others/Miscelleneous	Organs	Experimental Group	Occurrence
Steroid biosynthesis	Gills	LR/C	6
	Brain	LR/RR	
	Liver	LR/RR	
Steroid hormone biosynthesis	Gills	LR/C	3
	Intestine	LR/RR	
	Brain	LR/RR	
Drug metabolism cytochrome P450	Intestine	LR/C	2
	Brain	LR/RR	
Histamine (H3) receptor antagonists	Brain	LR/RR	1
Glutathione metabolism	Gills	LR/RR	4
	Brain	LR/RR	
	Liver	RR/C	
Glucocorticoid synthesis	Intestine	LR/RR	1
Pyrimidine metabolism	Gills	LR/C; RR/R; LR/RR	4
	Liver	LR/C	
Purine metabolism	Brain	LR/RR	3
	Liver	LR/C	
	Intestine	LR/C	

Note: LR/C – MCLR exposed vs. Control group; RR/C – MCRR exposed vs. Control group; LR/RR – MCLR vs. MCRR group

A steroid hormone is a steroid that acts as a hormone. Steroid hormones can be grouped into five groups by the receptors to which they bind: glucocorticoids, mineralocorticoids, androgens, estrogens, and progestogens. Steroid hormones help control metabolism, inflammation, immune functions, salt and water balance, development of sexual characteristics, and the ability to withstand illness and injury. These steroid hormones normally circulate as their sulphated derivatives or bound to carrier proteins, which have no hormonal effects; the free steroid is released only at the target tissue by the action of tissue-bound enzymes (O'Malley 1990). All steroids are fat-soluble and readily cross the cell membrane, interacting with dimeric receptor proteins. The steroid-receptor complex

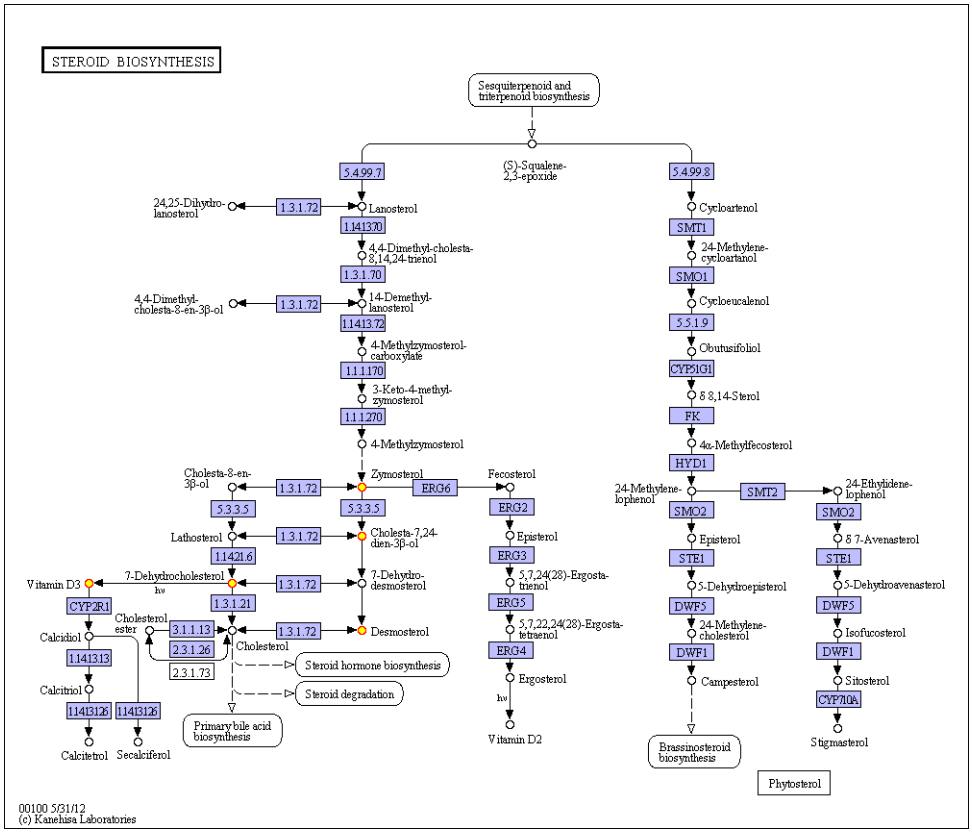


Figure 7.9: Steroid synthesis: Metabolites indicated as yellow solid circles were perturbed following an exposure to MCLR/MCRR in zebrafish gills

binds to target regions of DNA termed “response elements”. This activates the cascade of reactions, which are the response to the presence of steroids. Most of the male and female reproductive hormones belong to the class of steroid hormones. Perturbation of the metabolic pathways involved in their synthesis or action could have serious ecological consequences. A number of chemical compounds, known for their endocrine disrupting action (endocrine disrupters), can affect the action of these steroid hormones (Waring and Harris, 2005). Endocrine disrupters usually act as steroid agonists and bind to the receptor proteins. There have been recent reports where MCLR was observed to mimic an endocrine disruptor in human breast carcinoma cells and zebrafish larva. They observed weak estrogenic effects on larval zebrafish which was characterized by up-regulation of genes produced in the liver in response to estrogens (Oziol and Bouaïcha 2010; Rogers et al., 2011). Endocrine disruption from MCLR could extend throughout aquatic ecosystems, and also impact the terrestrial environment, including birds and mammals. Indirect effects on reproduction were also observed in some of the lipid and amino acid related pathways, as discussed in previous sections. It is evident from these set of preliminary experiments that MCs could extend a wide range of health effects on the organisms exposed with possible implications to all the important biological processes. Apart from exerting hepatotoxicity, MCs are could affect a lot of other key cellular processes as well which could be carried over through the entire ecosystem.

Apart from steroid hormone synthesis pathways, glutathione (GSH) metabolism was also impaired in the zebrafish organs following the exposure. GSH is one of most important and abundant antioxidant in the cells. GSH plays critical roles in protecting cells from oxidative damage and the toxicity of xenobiotic electrophiles, and maintaining redox

homeostasis (Forman et al., 2009). Elimination of many xenobiotic compounds can be accomplished through conjugation with GSH followed by secretion of adduct from the cell (Boyland and Chasseaud 1969). This conjugation often takes place with the help of antioxidant enzymes in the glutathione family like GST, GPx, GR. Published reports in the literature have confirmed the detoxification following the exposure with MCLR through a conjugation reaction aided by GST (Pflugmacher et al., 1998, Details in Chapter 5). In the previous chapter (Chapter 5), it was also shown that due to the balneation exposure to MCLR and MCRR, enzyme systems linked with GSH (GPx, GST,GR) have been affected, resulting in disturbed enzyme activities. Results from the present study are complementary to those obtained from biochemical studies (chapter 5) and suggest that oxidative stress is one of the adverse health impacts that follow the balneation mode of exposure to MCs.

Enzyme activities and GSH metabolism are inter-dependent on each other. If GSH is not abundant in the cells, molecular mechanisms would restrict the synthesis of the enzyme transcripts and vice-versa. In the present case, since the GSH metabolism was impaired, enzyme activities also decreased in some experimental groups as shown in Chapter 5. Decrease in antioxidant enzyme activities could have serious implications such as increased stress on zebrafish organs ultimately resulting in fatality or improper growth and development. Even though the concentrations of MCLR and MCRR are not sufficiently high to cause immediate fatal effects, they could alter the oxidative balance, which could lead to many deleterious effects and eventually, fatality if sustained for longer periods of time.

Purine and pyrimidine metabolism were also identified as the biochemical pathways that were perturbed following an exposure with MCLR/MCRR (Figure 7.91). Purine and pyrimidine are nucleoside bases that serve as backbone of nucleic acids like DNA (deoxyribonucleic acid) and RNA (ribonucleic acids). Nucleic acids contain genetic instructions used in the development and functioning. Alterations in the nucleoside bases could hold potential to interfere with the genetic machinery as well. Any damage to DNA could lead to lot of ecological implications. There are published reports in the literature that showed DNA strand breaks in human cell lines at doses that were not cytotoxic (lethal or fatal to cells). They suggested that DNA strand breaks are intermediates, produced during the cellular repair of MCLR induced DNA damage (Zegura 2003).

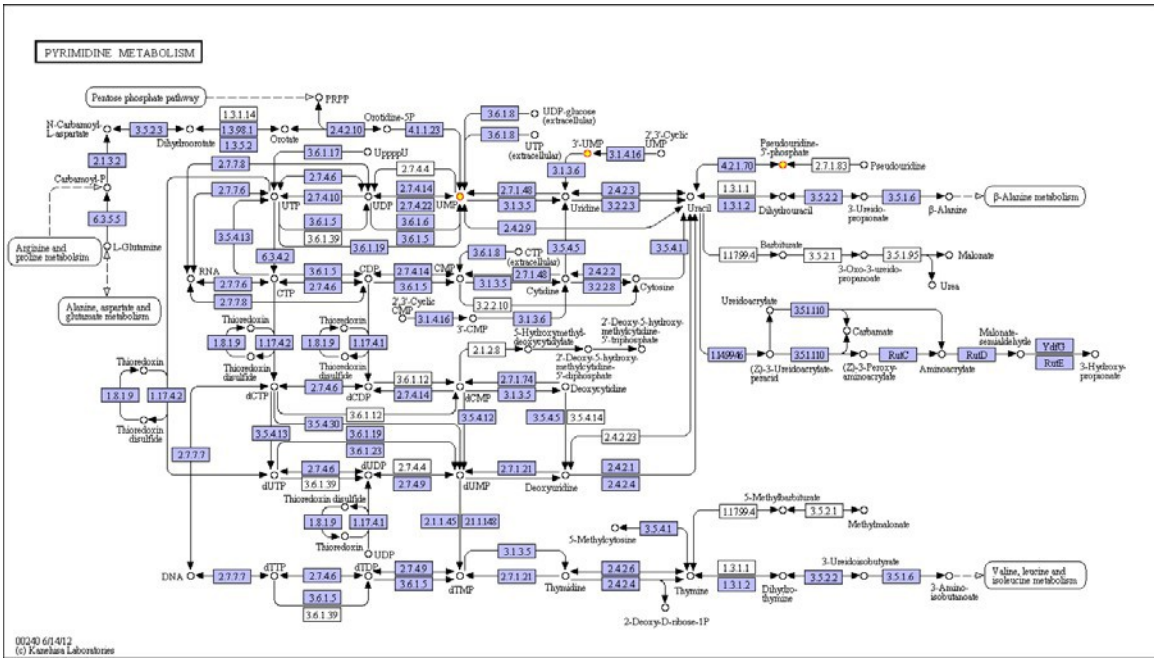


Figure 7.10: Pyrimidine metabolism: Metabolites indicated as yellow solid circles were perturbed following an exposure to MCLR/MCRR in zebrafish gills

There are reports on DNA damage in various other organisms including mouse, hamster etc (Rao and Bhattacharaya, 1996; Rao et al., 1998). Zegura et al., 2003 in their experiments also studied the mechanism of DNA damage and reported that MCLR induced both oxidised pyrimidines and purines. This supports the results obtained in the present set of experiments. Zegura et al., (2003) , further suggested that a substantial portion of the MCLR induced DNA strand breaks originated from excision of oxidative DNA adducts which might be a mechanism by which chronic exposure to low concentrations of MCs contribute to increased risk for cancer development (Zegura et al., 2003). This clearly indicates that sub-lethal doses of MCLR/MCRR found in balneation exposure could also lead to cancer and tumor promotion. Hence, sub-lethal doses of MCs could have very serious ecological implications considering the stability of MCs (as discussed in Chapter 2); MCs could sustain in the water bodies for longer periods causing a sub-lethal exposure to the sensitive organisms.

Apart from the above mentioned metabolic pathways, drug (cytochrome 450) and histamine (H3) antogonist receptor metabolic pathways were also identified to be perturbed following the exposure. The cytochrome P450 (CYP) superfamily is a large and diverse group of enzymes. The function of most CYP enzymes is to catalyze the oxidation of organic substances. The substrates of CYP enzymes include metabolic intermediates such as lipids and steroidal hormones, as well as xenobiotic substances such as drugs and other toxic chemicals (Guengerich, 2008). The role of CYP in MCLR/MCRR exposure has not been identified/studied by the scientific community earlier. Based on our study and previous reports on MCLR being an endocrine disruptor, where in, it could possibly act as a steroid agonist, it perhaps also activates the drug

metabolism pathway involving CYP since one of the substrates for CYP is steroids as mentioned above. Interestingly, CYP and steroid hormone metabolic pathways were both observed to be perturbed in organs exposed to MCLR. This could further support the explanation of MCLR activating the CYP drug metabolism pathway.

Histamine (H3) receptor antagonist pathway was also observed to be effected in case of MCLR exposed brain tissue in zebrafish. Histamine is an organic nitrogen compound involved in local immune responses as well as in triggering the inflammatory response. Histamine exerts its actions by combining with specific cellular histamine receptors (Hardie, 1989). There are four known histamine receptors, one of them is H3. H3 receptors control histamine turnover by feedback inhibition of histamine synthesis and release (West et al., 1990). A receptor antagonist is a type of receptor ligand that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses. H3-receptor antagonists are primarily found in the brain and are used to block the action of histamine on H3 receptors. Essentially, they enhance the activity of histaminergic neurons by blocking histamine receptors, thus preventing the detection of histamine by neurons impairing the feedback mechanism. Impairment of feedback mechanism causes the neurons to release more histamine, thus increasing their activity. Since histamine contributes to inflammatory response, production of more histamine by tissues would mean a severe inflammatory response against a foreign substance. There are reports in the literature that suggest the induction of ROS at the site of inflammatory response (Kehrer, 1993; Petrone et al., 1980). Thus, increased inflammation could lead to an increased production of ROS. ROS could further have a lot of implications on the organisms as discussed in Chapter 2 and 5. In the

present study, estimation of antioxidant systems suggested an increased flux of ROS following a balneation exposure to MCLR/MCRR. Inflammatory response could be one of the reasons for the increase in ROS. An in-depth understanding of the H3 antagonist receptor pathway could help in evaluating the possible reasons for MCs toxicity further.

The complete list of metabolites identified in each organ belonging to special class of metabolic pathways is given in the Supplementary section (Table S 1 to S 4). Metabolites were mainly identified in polar as well as non polar extracts of organs belonging to different experimental groups. Pathway maps for different organs (depicting the site at which a particular metabolite was perturbed, thus disturbing the whole pathway) are provided in the supplementary section.

Pathway maps presented here in this chapter are taken from KEGG software. KEGG is a database resource for understanding high-level functions and utilities of the biological system. These pathway maps are used to understand various biochemical and metabolic cycles as they occur in organisms. In the present study, metabolites identified from the polar and non polar extracts were mapped on KEGG. From the indentified processes and pathways, information on toxicity mechanisms and targets can be inferred.

7.4 Conclusion

The present study aimed at identifying the perturbation in key metabolites and the linked biochemical pathways following a balneation exposure to MCLR/MCRR. Zebrafish metabolome studies are still in their infancy, and the entire metabolome has not been fully characterized unlike zebrafish genome. This study represents the first attempt to understand the metabolite perturbations following an exposure to MCs. Since

metabolome is not well established, untargeted approach for identification was followed in the present study. Hence, there is a need for advance exploratory studies for confirmation of the identified metabolites obtained from this study.

Results from the present study could serve as background information for future metabolome studies pertaining to MCs exposure. Future studies could aim at target metabolites of interest as obtained from this set of global metabolite analysis, where in a total of 32 pathways got disrupted due to the perturbation in one/more metabolites in the entire chain. Since the metabolites are essentially the end products of gene expression, metabolites are better candidates to serve as biomarkers. mRNA, or gene expression may not give the full picture of what is happening inside the cell, and hence, including metabolite analyses for biomarker search is more appropriate. However, it is equally important to understand the changes occurring at gene levels. Integrating the data obtained from gene as well as metabolite levels could present deep insights into the mechanisms of toxicity exerted by MCs. This integration could further help in narrowing down the search for biomarkers and in establishing realistic risk management procedures

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CHAPTER 8

TRANSCRIPTOME CHANGES IN ZEBRAFISH ORGANS UNDER A BALNEATION EXPOSURE

This chapter describes the toxicological changes that occurred in zebrafish tissues after the exposure to MCLR/MCRR dissolved in water. After the exposure period, four tissues namely, gills, intestine, liver and brain were harvested from the zebrafish. After harvesting the tissues, RNA (nucleic acid, which controls gene expression and all processes within an organism) was extracted from them. Nucleic acids were then subjected to a series of processes which were done to evaluate and quantify the changes in RNA/gene expression that occurred in zebrafish tissues following the exposure to MCLR/MCRR as compared to control zebrafish tissues.

8.1 Introduction

From the previous set of experiments, it was evident that extracellular MCs are capable of causing adverse effects on zebrafish organs. However, the changes reported in the previous chapters are functional changes which may reflect the changes that occur at the molecular level. However, all the changes occurring at molecular levels may not result in any functional changes in terms of metabolite perturbation. Hence, molecular mechanisms may not present a holistic view of the impact of MCs on zebrafish organs following an exposure. However, owing to the limitations of the analytical and experimental techniques in metabolite detection and analyses, a global coverage of all the metabolites involved in biochemical pathways may not be completely feasible. Hence, it

is important to use an experimental approach that is complementary to metabolomics such that additional insights can be provided to understand the implications of balneation exposure of MCLR and MCRR in zebrafish organs at greater depths. Studying the changes in the zebrafish organs at the molecular level would best complement the results obtained from metabolite analyses. It is also important to study the changes occurring at the molecular level, i.e., gene expression or transcriptome, to understand in-depth mechanisms underlying the toxin action. The transcriptome can be defined as the complete set of transcripts in a cell and their quantity in a specific developmental stage or physiological condition. Transcriptomics aims to quantify the changing expression levels of each transcript during development and under different conditions. For environmental studies, data on transcriptomics could then be used for identification of potential biomarkers, or to reveal mechanistic aspects of health impacts posed by any environmental pollutant. With the advent in advanced experimental techniques, high-throughput transcriptomic search has been possible through use of gene expression microarrays.

Gene expression microarrays have been very useful to provide an overall view of how gene expression changes between two or more biological conditions (Croze, 2010). Microarray-based transcription profiling is now a consolidated methodology, and large-scale microarray studies have become a crucial aspect of a new way of conceiving experimental biology. Microarrays and their applications have been extensively described in literature (Nguyen, 2002, Allison et al., 2006). The microarray technology has been developed in several variants. However, for the present study, we have used one color (or oligonucleotides or one-channel) microarrays. In one channel microarrays, the

RNA of one sample is labeled with a fluorescent dye and hybridized to a single array where millions of copies of short (around 24 base pairs) oligonucleotide probes representing all known genes (several probes for gene form a "probeset") have been synthesized. After exposition to laser light and scanner, the intensity of each molecular probe location is measured yielding a value which represents an absolute measure of expression. These data from the gene expression microarray could give a lot of information on toxic response and toxicity mechanisms in various organisms. Molecular (gene expression) and metabolic data, together, could also help in narrowing down the list of potential candidates for biomarkers since all the changes at the molecular level might not transcribe into sensible information, and further may not have any functional role to play in terms of metabolic changes or behavior patterns.

A number of studies have been conducted on gene expression analyses with multiple organisms, cells including microarray investigations with adult zebrafish following an intraperitoneal injection with MCLR and MCRR (Yea et al., 2001; Li et al., 2009; Wei et al., 2008;Yan et al., 2012; Zegura et al., 2008 and Rogers et al., 2011). These studies revealed that numerous genes involved in immune function, tumorigenesis and cell cycling were differentially regulated in liver tissue. Zegura et al., (2008) investigated the role of MCLR on HepG2 cells. They found that found a significantly elevated expression of tumor suppressor gene and its downstream-regulated genes involved in DN repair and cell cycle regulation (Zegura et al., 2008). Li et al., (2009) investigated the role of MCRR on *Synechocystis sp*. They found that MCRR increased the gene expression of

antioxidant enzymes and cell aggregation, thus suggesting the possible role of oxidative stress in toxicology of MCRR (Li et al., 1999).

In larval zebrafish exposed to MCLR, immune-related genes and heat shock proteins were also differentially expressed in targeted analyses (Wei et al., 2010; Yan et al., 2010). Rogers et al. (2011) also published a comprehensive study on global gene expression profiling in larval zebrafish exposed to MCLR and *microcystis*, which revealed endocrine disrupting effects of MCs (Rogers et al.,2011). To our best knowledge, there has been no detailed gene expression study on extracellular MCs at sub-lethal concentrations. A systematic gene expression study with extracellular MCs is needed to gain a better understanding of the toxicological targets and mechanistic aspects of toxicity by using environmentally-relevant concentrations of MCs.

The present sets of experiments were conducted to analyze the global gene expression patterns using a high-throughput microarray to study RNA expression (Transcriptomics). Zebrafish were exposed to MCLR and MCRR (10.0 µg L^{-1}) under balneation conditions for 30 days. After the exposure, fish organs were harvested for the RNA extraction and subsequent microarray analysis.

8.2 Methodology

8.2.1 Fish exposure and sampling

Chemicals, reagents used for these experiments are given elsewhere in Chapter 3 (section 3.2.1, 3.2.2). Details about selection and procurement of fish are given out in Chapter 3 (section 3.4.1 and 3.4.2), including aschematic on experimental design of exposure for these studies (refer to Figure 3.4, Chapter 3).

Fish were exposed to MCLR and MCRR dissolved in aquarium water at a concentration of 10 µg L^{-1} for a period of 30 days. Briefly, stock solutions of MCLR and MCRR were prepared (10 mg L^{-1}) and were diluted to obtain the required concentrations. Water in the tanks was replaced with fresh water, spiked with the toxins daily. Dosing concentrations were confirmed by measurements using liquid chromatograph, composed of an HP100 liquid chromatograph (Agilent Technologies, U.S.A) interfaced with a triple quadrupole MS/MS (Applied Biosystems, U.S.A). Analytical separation was achieved on a Zorbax Extend-C18 5 µm, 2.1 x 150 mm (Agilent technologies, Germany). The injection volume was 10 µL. The mobile phase consisted of 0.1 % formic acid (solvent A) and methanol (solvent B). A gradient elution was used, starting with water: methanol at 90:10 from 0 to 6 min, and switching to 5:95 up to 10 min before returning to the original conditions to re-equilibrate the system (As done for biochemical experiments, chapter 5).

The capillary voltage was set at 89 volts and the cone voltage at 4 volts. The desolvation gas (nitrogen) temperature and flow-rate were set at 350°C and 615 L/h, respectively. The ion source temperature was set at 120°C. LC-MS-MS was operated in the positive ion mode. MCLR and MCRR were monitored by using the MS instrument in the SRM mode: m/z 995.6; fragment ion at 135.1 and m/z 520; fragment ion 135.1 respectively. At the end of exposure period, fish (n= 5 per group) were sampled out and the organs (gills, liver, intestine and brain) were harvested, snap frozen and stored at -80ºC until RNA extraction (Refer to figure 3.8, section 3.5.5; Chapter 3). Kits and chemicals used for the further sample preparations are given in Chapter 3, section 3.6

8.2.2 RNA extraction

Prior to RNA extraction, two tissue samples were pooled together to form one biological replicate. This was done to ensure that enough RNA sample was available for hybridization on the microarray. Total RNA was extracted from harvested frozen tissues using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The integrity of RNA samples was verified by using bioanalyzer (Agilent technologies), and the concentrations were determined by Nanodrop UV spectrophotometer. Details are given in Chapter 3, section 3.5.5.

8.2.3 cRNA synthesis and Purification

Following RNA extraction and quality analyses, RNA samples from tissues were converted to cDNA. cDNA was then transcribed to cRNA and amplified using a transcription mix. The cRNA samples were then purified and quantified on a nanodrop. Quality control check of the purified cRNA was done according to the manufacturer's instructions. Briefly, cRNA was quantified using nanodrop (ND-1000-UV-VIS) spectrophotometer.

Concentration of cRNA was used to determine the yield of cRNA (µg cRNA) as follows:

(*Concentration of cRNA*) * 30 µL (*elution volume*) / 1000 = µg of cRNA Eq(8)

Concentrations of cRNA (ng/µL) and cyanine 3 (pmol/µL) were used to determine the specific activity as follows (Eqn 9):

(*Concentration of Cy3*) / (*Concentration of cRNA*) * 1000 = pmol Cy3 per µg cRNA

if the yield was <1.65 µg and the specific activity was < 9.0 pmol Cy3 per µg of cRNA,

then entire process of cRNA preparation was repeated. The samples that passed the quality control check were used for hybridization.

8.2.4 Hybridization and scanning

Quantified cRNA samples were then hybridized onto microarrays following the manufacturer's instructions and incubated in a chamber under optimized settings. Later, the microarrays were washed sequentially with different buffers and then scanned for their intensities using a scanner. Following the scan, information on features was extracted from microarray scan data which allowed measuring gene expression in the experiments. This was done with the help of software integrated with the scanner.

8.2.5 Data analyses

After feature extraction, the data were exported to Genespring software for analyses. Genespring was used for mining, normalization, visualization, statistical analyses of data obtained from microarray experiments (Gene expression studies). Data files containing the gene expression data from zebrafish tissues exposed to MCLR and MCRR along with controls were first imported to Genespring user interface in particular file format. They were then grouped depending upon various experimental parameters. This particular grouping was done under "Interpretation" module of Genespring. An interpretation defines a particular way of grouping samples into experimental conditions for both data visualization and analyses. The entity or data lists along with the particular interpretation were filtered first by expression then filtered by flags. After the filtering, data were used for significance analysis. In the present case, one-way ANOVA ($p<0.05$) was used as a test of significance. The entity list/ gene list obtained from significance analysis was then

subjected to fold-change analysis. The gene list was then visualized and plotted using different tests. Lastly, the gene list was imported to pathway mapping module to understand the biochemical pathways that were affected by those gene transcripts.

8.3 Results and Discussion

Global gene expression analyses were done on zebrafish tissues after a 30 day balneation exposure with MCLR and MCRR (10 µg L^{-1}). A total of 2430 genes were significantly affected upon exposure to MCLR/MCRR ($p<0.05$, >2-fold change). Out of 2430 genes, 2354 genes were significantly affected in gill tissues; 23 being affected in brain and 53 being affected in intestine tissues. A list of the genes with their annotations is given in supplementary data Tables (S 5-8). It is interesting to note that MCRR affected a more number of genes in the case of intestine and gill tissues of zebrafish as compared to MCLR. A total number of 52 and 1822 genes were affected after exposure with MCRR as against 12 and 1137 genes affected by MCLR in gills and intestine, respectively. In brain, however, MCLR exposure disturbed the gene expression of 20 genes as compared to 18 gene transcripts as observed with MCRR exposure. It was observed that 15, 11, 605 genes transcripts were common between MCLR and MCRR exposed zebrafish brain, intestine and gill tissue (Supplementary Tables 5-8). The possible reason for this observation could be the difference in toxic potencies and the biological affinities for different sites within the zebrafish organs for MCLR and MCRR. Variations in the toxic potencies and biological affinities could lead to differences observed in gene expression patterns in zebrafish organs exposed to MCLR and MCRR.

The altered genes transcripts, as observed in this experiment, would finally be translated into an amino acid sequence which would have a functional role in the biochemical cycles. Any change in the gene transcript in terms of its regulation could, thus, impair and affect all the biochemical pathways, which could finally lead to disruption of entire metabolism. Understanding the biochemical pathways that are affected through the MCLR/MCRR exposure could provide further insights into mechanisms of toxicity. Hence, after searching for significant differences in gene transcripts, the data sets were imported into pathway mapping module in Genespring, as mentioned in one of the previous sections.

After doing the search, a number of pathways were observed to be affected as a result of up/down regulation of gene transcripts. In some of the biochemical pathways as many as 10-12 gene transcripts were affected. More number of pathways was deranged in gill tissues of zebrafish as compared to those in other organs. This trend is consistent with that observed for the number of gene transcripts in different organs, for example, the maximum number of gene transcripts were affected in gill tissues. Moreover, metabolite analyses also show perturbation in a larger number of metabolites in gills exposed to MCLR/MCRR than those in other organs (for details, Chapter 7). This observation could be attributed to the mode of exposure of the zebrafish to the toxins, which get absorbed primarily through gills during oxygen exchange (Figure 8.1). Very few detailed studies have been reported in the literature on gene expression profiling upon exposure of zebrafish to MCLR/MCRR (Wei et al., 2010; Yan et al., 2010). Most of these studies focused on acute exposure assessments (Li et al., 2009; Rogers et al., 2011), which are different from the chronic exposure studies undertaken in this doctoral study. In addition,

the administration of MCs to the fish as addressed in the previous studies is also different from the one in the present study.

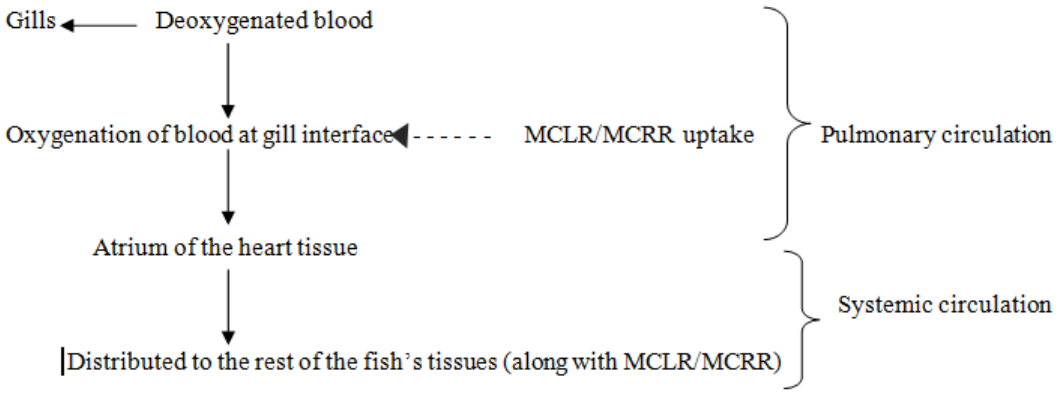

Figure 8.1: MCLR/MCRR distribution and uptake kinetics in zebrafish tissues for present study

Effects of MCLR/MCRR exposure on biochemical pathways

8.3.1 Brain Tissue

As mentioned earlier, a total of 23 genes were affected in brain, out of which; 15 gene transcripts were significantly up/down regulated as compared to controls in the case of both MCLR and MCRR exposed tissues. Among the rest, 3 gene transcripts were only affected in the case of MCRR exposure while 5 gene transcripts were exclusively observed in the case of brain tissues exposed to MCLR (Figure 8.2, 8.3). As mentioned earlier, these gene transcripts could affect one or several biochemical pathways

depending upon the functional role of the amino acid sequences they code for. A search on pathway mapping module revealed that 11 pathways got affected due to the changes in gene expression (Table 8.1).

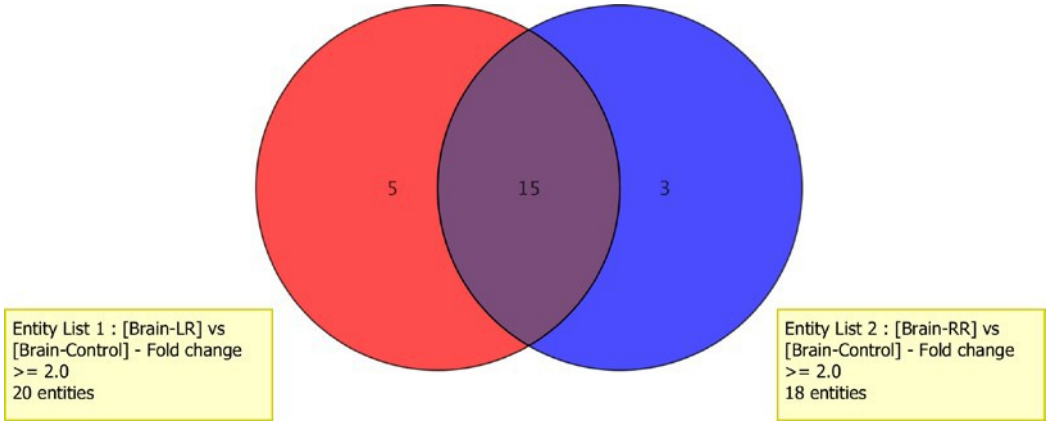

Figure 8.2: Venn diagram showing the distribution of significant gene transcripts in brain tissues exposed to MCLR/MCRR ($p < 0.05$ (corrected), fold change >2)

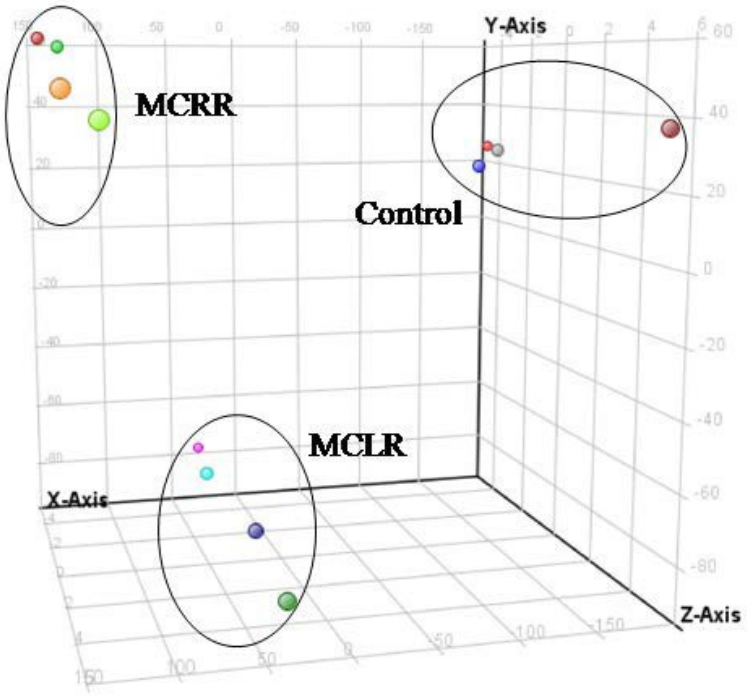

Figure 8.3: PCA plot showing the clustering of the three groups namely control, MCLR treated and MCRR treated brain tissue gene transcripts

Table 8.1:

Affected biochemical pathways in brain tissues upon exposure

Pathway	Matched Gene entities
Dr_ERK1_-_ERK2_MAPK_cascade_WP402_40778	4
Dr_FGF_signaling_pathway_WP152_40834	4
Dr_TGF-beta_Receptor_Signaling_Pathway_WP1367_40788	4
Dr_MAPK_signaling_pathway_WP1337_47927	3
Dr_EGFR1_Signaling_Pathway_WP1323_40845	2
Dr_Oxidative_Stress_WP1372_40812	2
Dr_Myometrial_Relaxation_and_Contraction_Pathways_W	2
Dr_Insulin_Signaling_WP1313_47923	1
Dr_TGF_Beta_Signaling_Pathway_WP1370_40848	1
Dr_Selenium_metabolism_Selenoproteins_WP1358_40805	1
Dr_estrogen_signalling_WP1330_46427	1

Most of these biochemical pathways belong to cell signaling which are involved in key cellular processes for both the adult organisms and the developing embryos including cell growth, cell differentiation, apoptosis, cellular homeostasis and other cellular functions (Kholodenko, 2006). The transforming growth factor β (TGF-β) family plays an important role in tumor suppression, cell proliferation, cell differentiation, tissue morphogenesis, lineage determination, cell motility and apoptosis (Massague, 1998). The Fibroblast growth factor (FGF) pathway is also involved in cell differentiation, cell migration, cell shape and cell proliferation (Figure 8.4, 8.5). Similarly, mitogen-activated protein kinases (MAKP) pathways are also involved in regulation of cell death through apoptosis. Komatsu et al. (2007) reported that MCLR activated several MAPKs including ERK1/2, JNK, and p38 through inhibition of protein phosphatases; these species are involved in processes like proliferation, gene expression, mitosis, cell survival and apoptosis. Komatsu et al., (2007) used low doses of MCLR on human embryonic kidney cells (HEK293) and demonstrated that MCLR induces apoptosis through activation of multiple MAPK pathways (Komatsu et al., 2007). Results obtained by Komatsu et al. (2007) are consistent with those observed in this study in that, MAPK signaling in zebrafish tissues as well (Figure 8.6). All these biochemical pathways indicate that exposure to MCLR/MCRR in brain tissues could eventually lead to tumor promotion. The main effect of a tumour promoting agent is its ability to induce an alteration of gene expression, resulting in aberrant cell differentiation, proliferation and apoptosis through intervention with various cell signalling pathways (Hsiang et al., 2004; Chen et al., 1998).

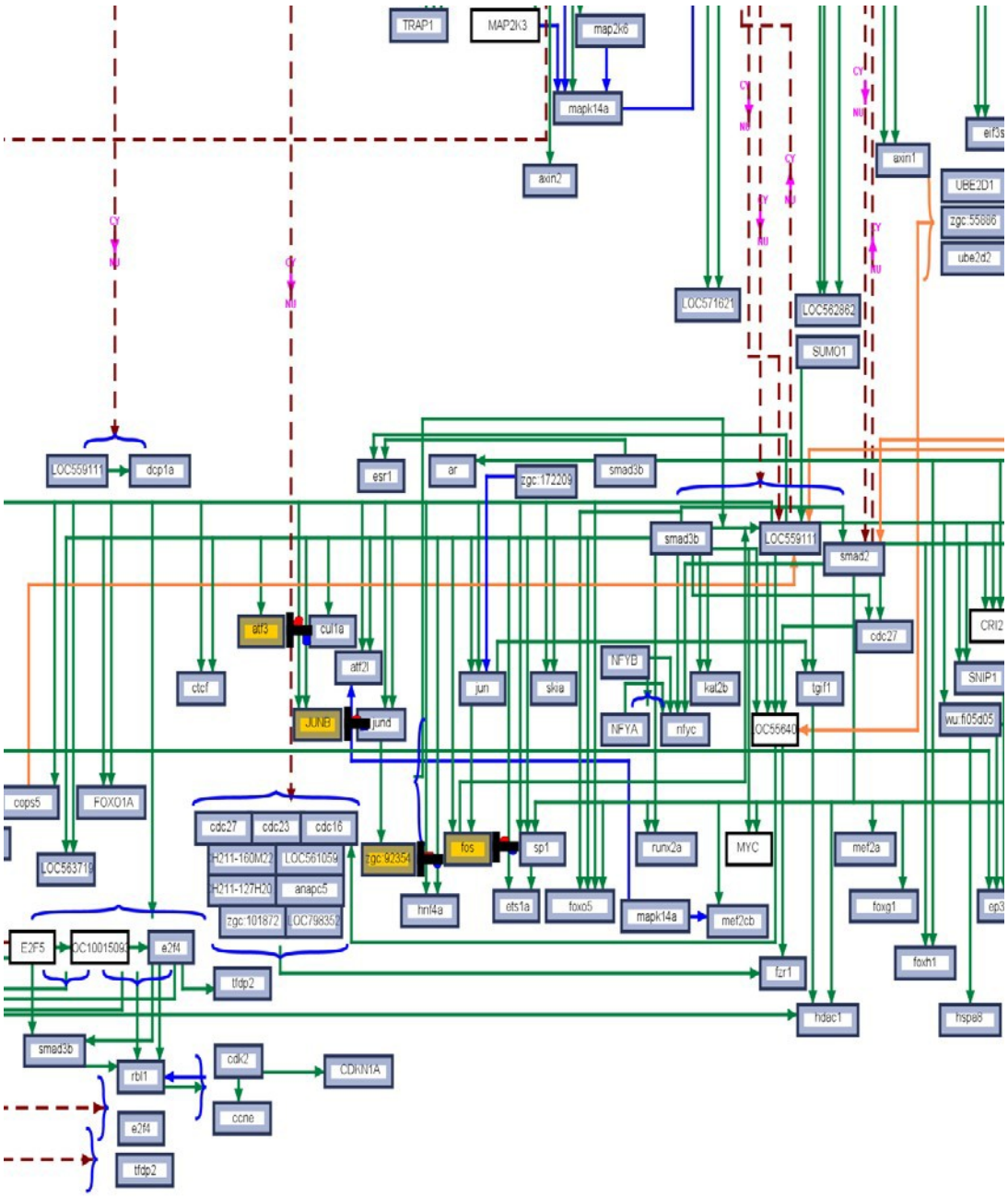

Figure 8.4: Dr_TGF-beta_Receptor_Signaling_Pathway_WP1367_40788 in zebrafish brain tissues exposed to MCRR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish brain tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCRR exposed gene transcripts)

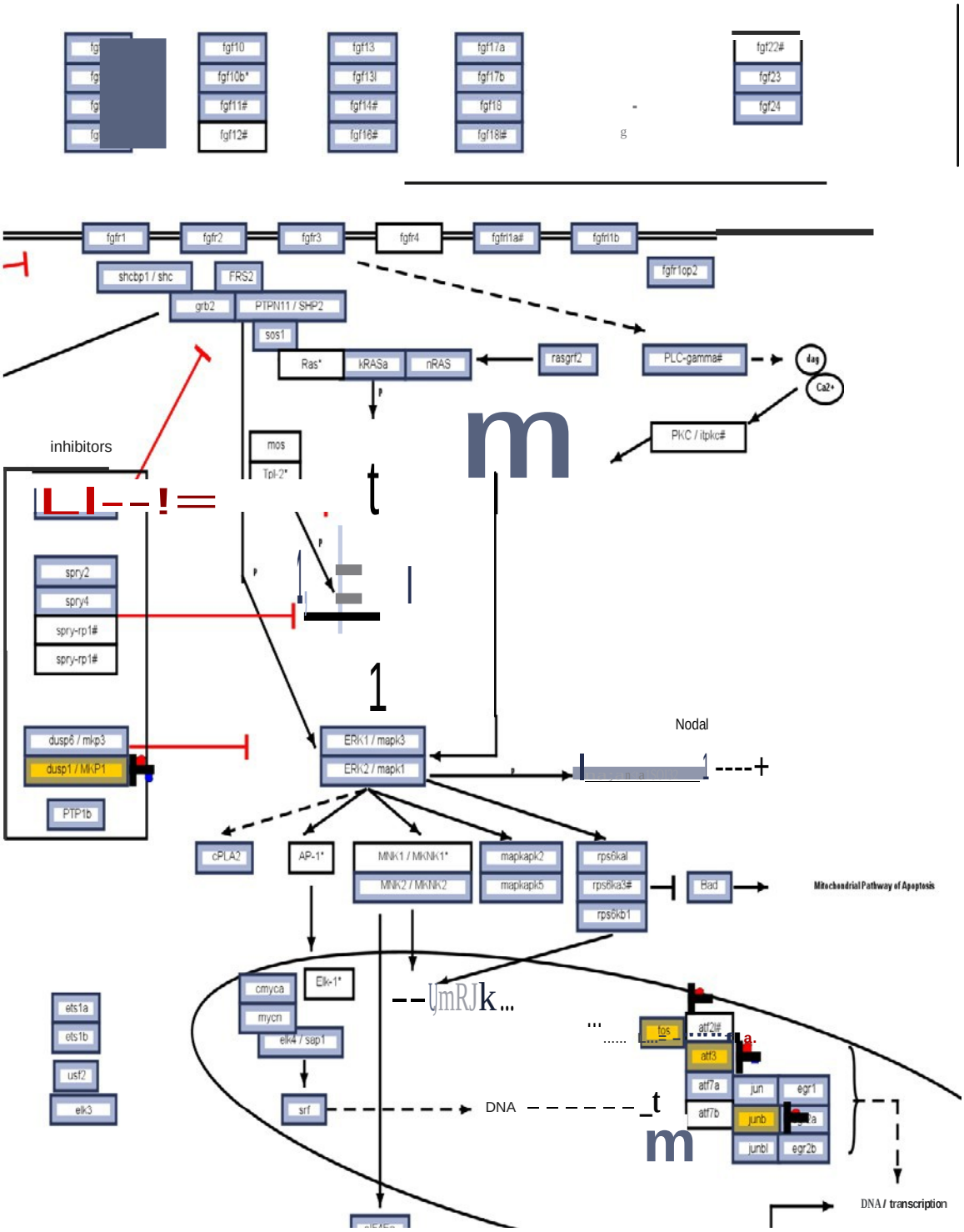

Figure 8.5: Dr_FGF_signaling_pathway_WP152_40834 in zebrafish brain tissues exposed to MCLR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish brain tissues (indicated in the bar next to the gene transcript; ▬ intensity of control gene transcripts, ▬ intensity of MCRR exposed gene transcripts)

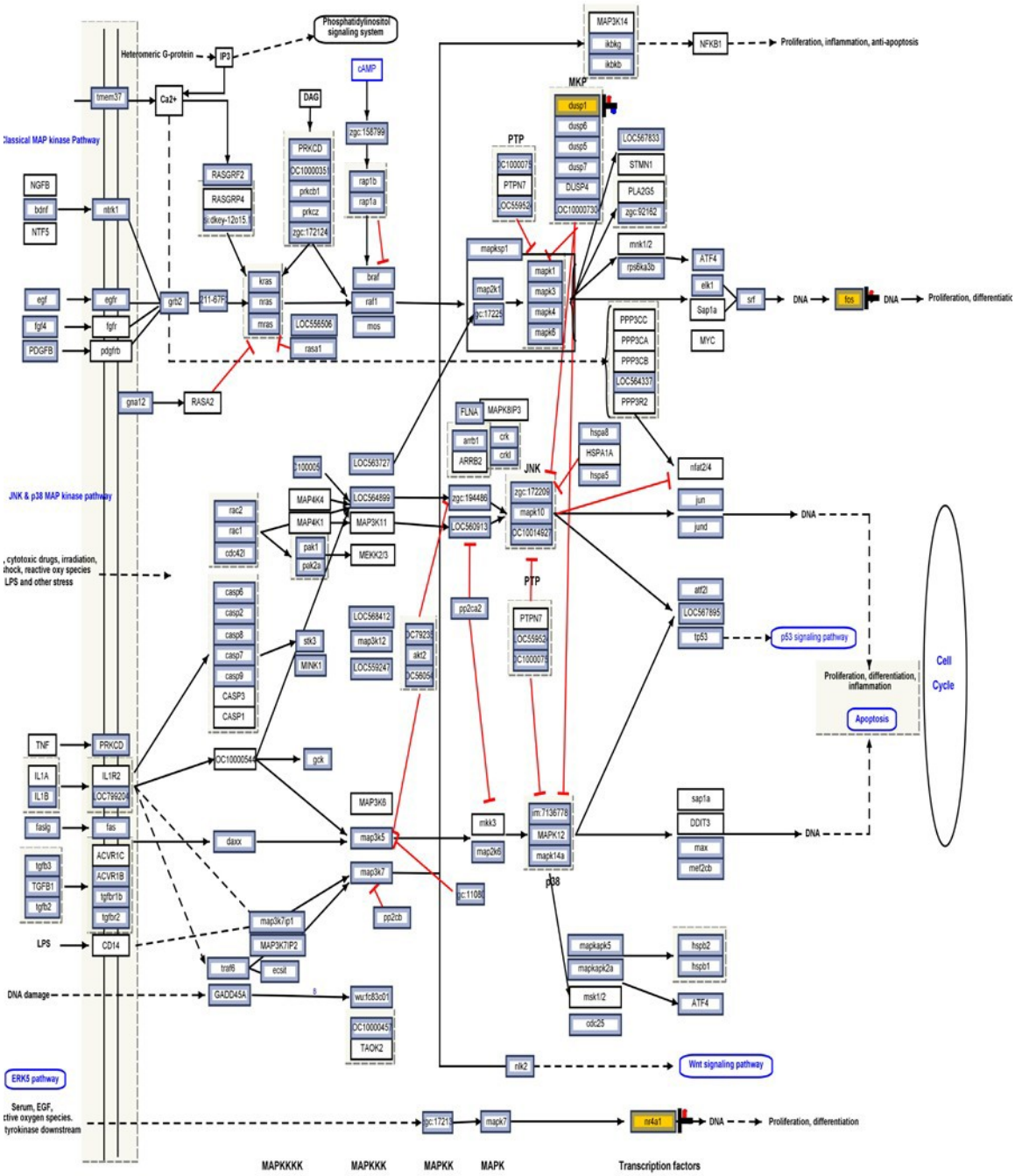

Figure 8.6: Dr_MAPK_signaling_pathway_WP1337_47927 in zebrafish brain tissues exposed to MCRR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish brain tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCRR exposed gene transcripts)

An abnormal activation of the MAPK signalling pathway, controlling cell growth and apoptosis, etc. is common in tumors, which could promote cell proliferation (Aguirre-Ghiso et al., 2003; Ostrowski et al., 2000). MCLR has been known for tumour promotion due to its tumour promoting effect (Gehringer, 2004). Our study has shown similar effects for MCRR exposed brain tissues as well. In the event of a cyanobacterial bloom lysis, there is a high likelihood for MCLR and MCRR to be present together in the extracellular phase. In such an event, fish would be exposed to both MCLR and MCRR. From the results obtained in present study, it could be speculated that MCLR and MCRR may work in synergism if present, at the same point. This synergism could further intensify the toxic implications of MCLR/MCRR exposure to fish.

Apart from the cell signaling pathways, oxidative stress pathways were also affected by both MCLR/MCRR exposure due to deregulation in gene transcripts as shown in Table 8.1. Oxidative stress is classically defined as a disturbance in the pro-oxidant/antioxidant balance in favor of the former, leading to potential molecular damage (Halliwell and Gutteridge, 2007), as mentioned in previous chapters. MCs uptake has been related to the production of ROS, leading to an increase in lipid peroxidation and DNA damage (Details in Chapter 2, section 2.6).

Production of ROS leading to an increase in oxidative stress after balneation exposure has been discussed in one of the previous chapters on biochemical enzymes (Chapter 4). Metabolic analyses showed that glutathione metabolism was perturbed in brain tissues as a result of balneation exposure to MCLR/MCRR (Chapter 7). Observations made from the current set of experiments further strengthen the results obtained from other

biological platforms employed in the present study, and suggest that balneation exposure to extracellular MCs could also lead to oxidative stress in zebrafish organs.

Insulin signaling pathways also appear to be deregulated as a result of MCLR/MCRR exposure onto zebrafish brain tissues. Insulin hormone production is related to glucose metabolism through glycolysis. Glucose metabolism was perturbed in brain tissues as a result of MCLR/MCRR exposure (details in Chapter 7). Deregulation of insulin signaling pathways seems to be associated with deranging of glucose related metabolic pathways. MCs are known to be inhibitors of protein phosphatases, which play a key role in glycogen metabolism (Moses et al., 1972), and these enzymes could also indirectly affect glucose metabolism, which is observed in this present study. The brain metabolizes glucose as its main energy source, and disturbances in glucose transport can be debilitating or fatal (Sokoloff, 1973). This glucose metabolism implies that even low doses of MCLR/MCRR in a chronic expsoure can eventually lead to fatal consequences among exposed organisms such as fish.

Estrogen signaling pathways were also observed to be perturbed which eventually affected the steroid hormone synthesis metabolism as seen with metabolite analyses. Male zebrafish specimens were used in all the experiments in this thesis and hence, activation of estrogen linked pathways suggests the possible endocrine disruption ability of MCLR/MCRR in a chronic exposure episode. This biochemical change leading to endocrine disruption could eventually result in a massive ecological imbalance. It could possibly lead to reproductive abnormalities. From an ecological viewpoint, there is concern that adverse effects on reproduction in individuals might lead to population-

level effects thus causing a disturbance in the entire ecosystem (Taylor and Harrison,1999) .

Lastly, selenoproteins metabolism was also affected due to the deregulation of gene transcripts in brain tissues. Interestingly, perturbation of selenoamino acid pathways was observed in brain tissues as a result of MCLR/MCRR exposure as discussed in Chapter 7. Results from the present experiments support are consistent with the findings from metabolite analyses, suggesting that any disturbance in gene expression can lead to consequent perturbation at the metabolic functional level.

It is clear from the above mentioned results that a lot of adverse effects observed at the metabolic level upon exposure of zebrafish to MCLR/MCRR have their roots at the molecular level as shown from the up/down regulation of gene transcripts. Interestingly, a lot of other cell signaling pathways were also observed to be deregulated at the molecular level, which was not identified at the metabolic level in the present set of experiments.

8.3.2 Intestine

As mentioned earlier, a total of 53 genes were significantly affected in intestine, out of which, 11 gene transcripts were significantly up/down regulated as compared to controls in case of both MCLR and MCRR exposed tissues ($p < 0.05$ (corrected), fold change >2). 41 gene transcripts were only deregulated in the case of MCRR exposure while only 1 gene transcript was exclusively seen in the case of brain tissues exposed to MCLR($p < 0.05$ (corrected), fold change >2) (Figure 8.7 - 8.9). The variations observed could be due to difference in the biological affinities as explained in the earlier section on Brain

tissues in this chapter. A search on pathway mapping modules revealed that 5 biochemical pathways were affected due to the changes in gene expression (Table 8.2).

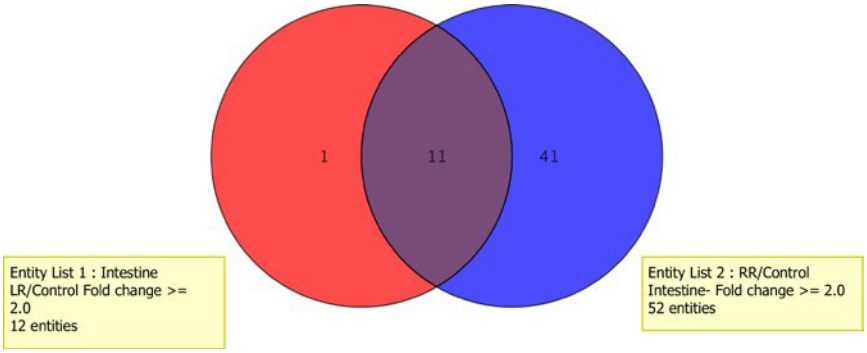

Figure 8.7: Venn diagram showing the distribution of significant gene transcripts in intestine tissues exposed to MCLR/MCRR (p <0.05 (corrected), fold change >2)

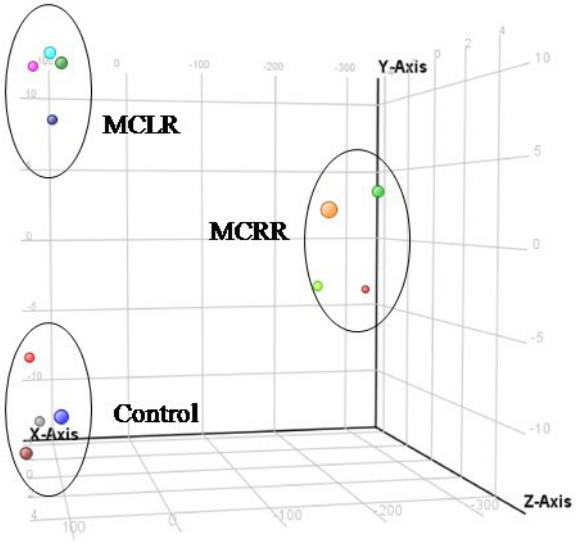

Figure 8.8: PCA plot showing the clustering of the three groups namely control, MCLR treated and MCRR treated intestine tissue gene transcripts

Table 8.2

Affected biochemical pathways in intestine tissue upon exposure

Pathway	Matched gene entities
Dr_Eukaryotic_Transcription_Initiation_WP344_40863	1
Dr_Electron_Transport_Chain_WP1339_35269	1
Dr_TNF-alpha_NF-kB_Signaling_Pathway_WP1369_40798	1
Dr_Oxidative_phosphorylation_WP1335_40841	1
Dr_mRNA_processing_WP467_40828	1

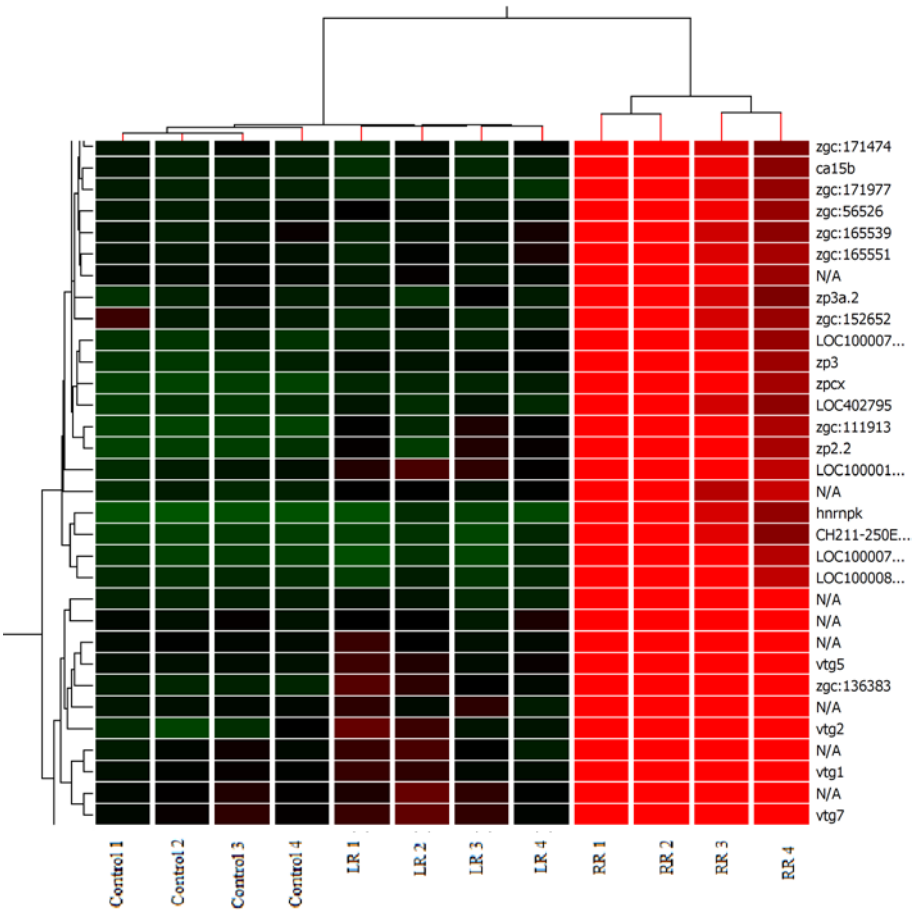

Figure 8.9: Heat map showing the difference in gene expression in a selected cohort of transcripts between MCRR and control group, intestine tissues (■ Minimum intensity of gene probe;■ Medium intensity of the gene probe;■ Maximum intensity of gene probe)

The first pathway hit detected by the software module was eukaryotic transcription (process of creating a complementary RNA copy of a sequence of DNA) initiation (Figure 8.10). The regulation of gene expression is achieved through the interaction of several levels of control including the regulation of transcription initiation. If the transcription initiation signaling pathways are affected, they could lead to up/down regulation of genes resulting in functional abnormalities at protein and metabolite levels. From metabolite analyses, it became evident that metabolism in intestine tissues was affected following a balneation exposure with MCLR/MCRR. Disruption at metabolic levels is a result of regulatory changes at gene expression and molecular levels. mRNA processing was also observed to be affected by the exposure of MCLR/MCRR to intestine tissues. Transcription is followed by processing of RNA in different stages. Any disturbance in transcriptional processes would further affect translation (generation of protein from mRNA sequence), thus impairing the metabolic machinery at different levels.

The software also detected two interlinked processes: oxidative phosphorylation and electron transport chain; gene transcripts involved in these two processes were affected as a result of MCs exposure (Figure 8.11). Oxidative phosphorylation is a metabolic pathway that uses energy released by the oxidation of nutrients to produce adenosine triphosphate (ATP) (Berg et al., 2002). During oxidative phosphorylation, electrons are transferred from electron donors to electron acceptors such as oxygen, in redox reactions. These redox reactions release energy, which is used to form ATP. These redox reactions

are carried out by a series of protein complexes called electron transport chains.

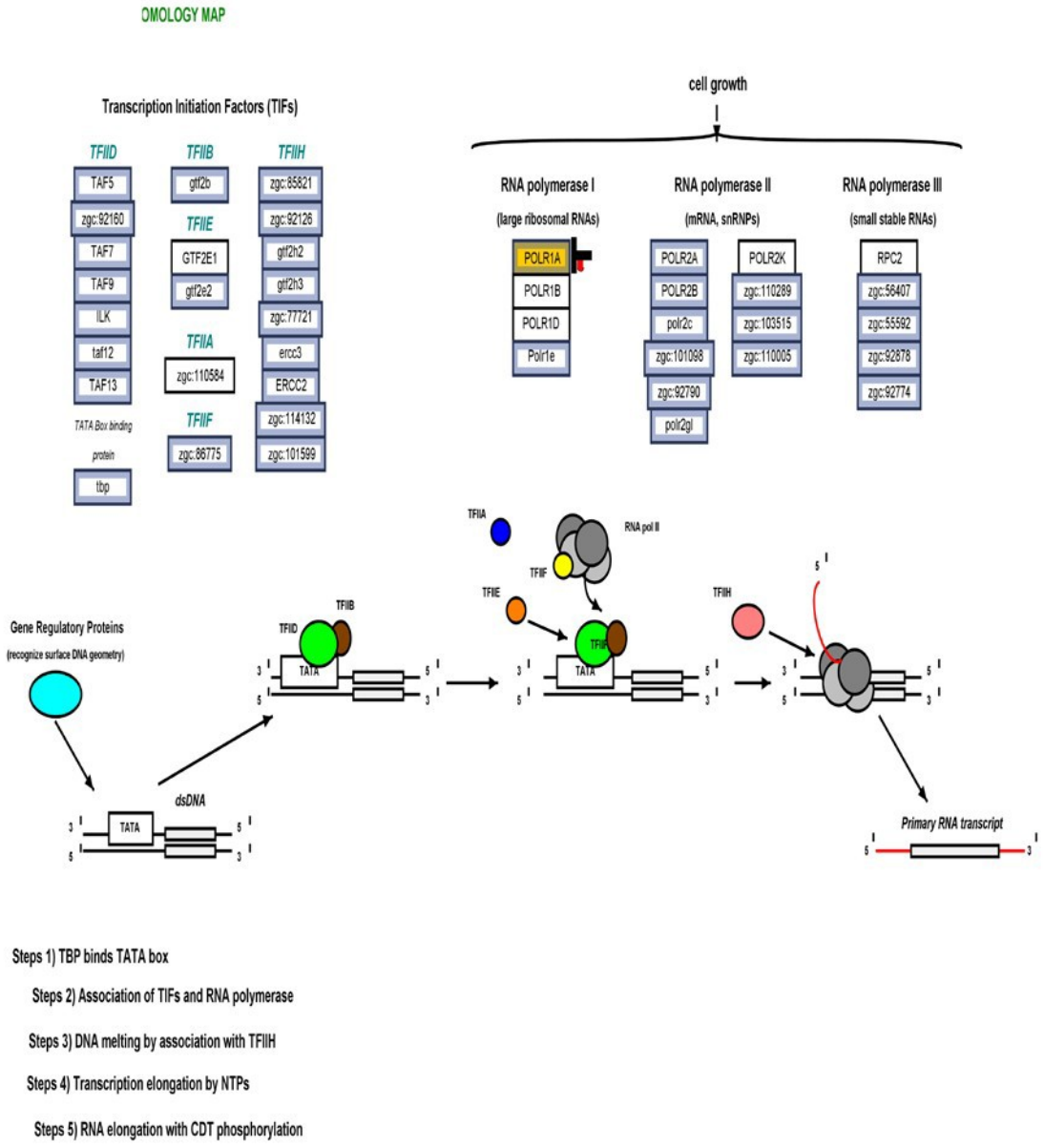

Figure 8.10: Dr_Eukaryotic_Transcription_Initiation_WP344_40863 in zebrafish intestine tissues exposed to MCLR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCLR exposed gene transcripts)

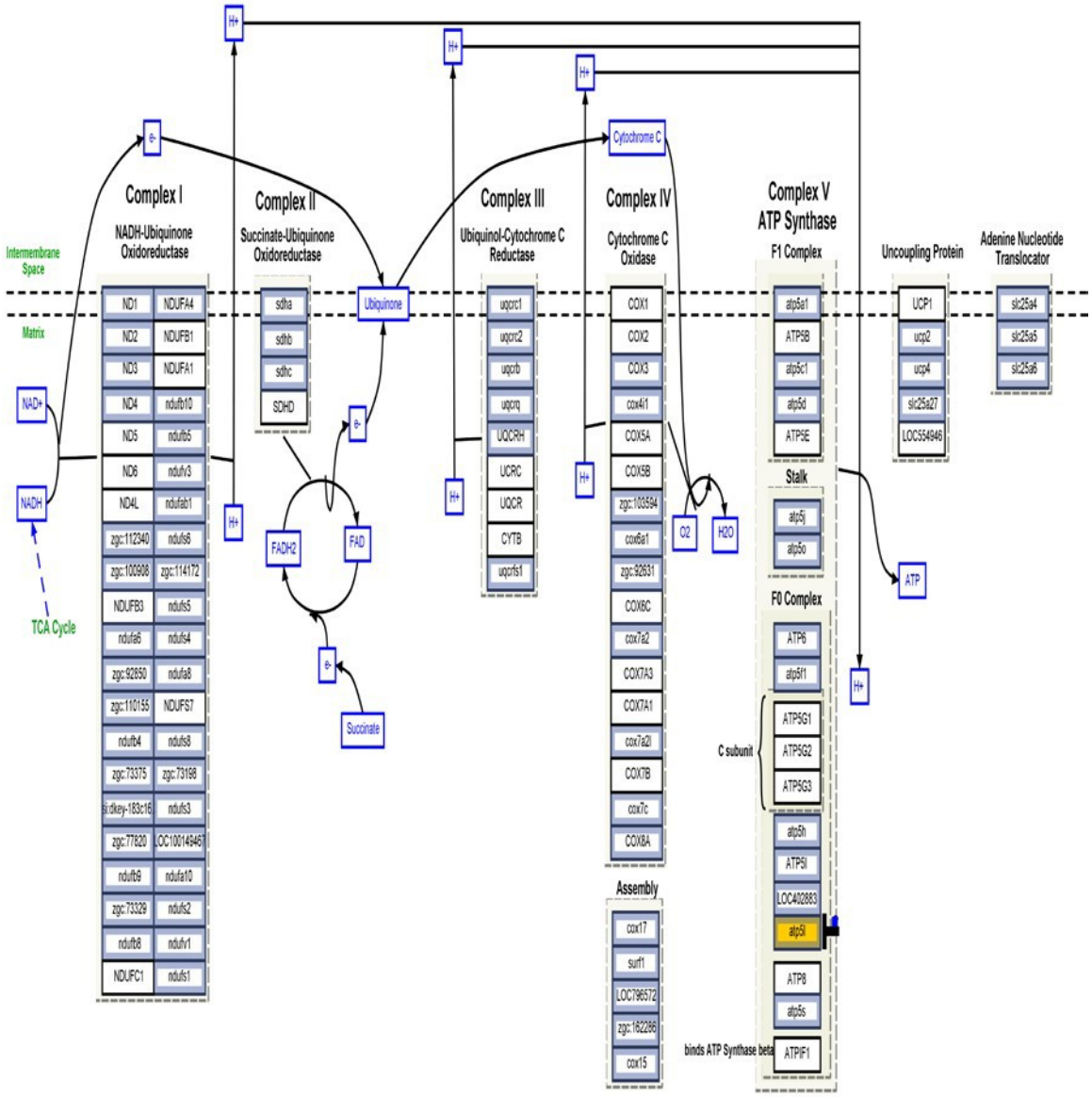

Figure 8.11: Dr_Electron_Transport_Chain_WP1339_35269 in zebrafish intestine tissues exposed to MCRR. Gene transcripts (marked in yellow) are significantly up regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCRR exposed gene transcripts)

Recent evidences suggest the role of oxidative phophorylation in tumour cells and their growth (Funes et al., 2007). Funes et al. (2007) showed that the transformation of human mesenchymal stem cells into cancer cells increases their dependency on oxidative phosphorylation for energy production, hence suggesting their role in tumorigenesis (Funes et al., 2007). The analyses of the metabolic changes that occur during the transformation of adult mesenchymal stem cells revealed that their dependency on oxidative phosphorylation was increased (Funes et al., 2007). It is also proven that alteration of oxidative phosphorylation by mutations in mtDNA (mitochondrial DNA) increases tumorigenicity in different types of cancer cells (Petros et al., 2005; Ishikawa et al., 2008). MCs are known to be tumor promoters (as discussed in Chapter 2); their ability to promote tumorigenesis in healthy cells requires the change in metabolic machinery to accommodate the increase in energy demands following tumorigenesis. Induction of tumor formation would cause destabilization in signaling pathways linked to energy production in cells as discussed above. From the results, it could be deduced that even balneation exposure to MCs could lead to tumor production and promotion in healthy cells in intestine tissues. A cell signaling pathway related to tumor promotion was also observed to be affected by the gene transcripts in intestine tissues. This signaling pathway was TNF (Tumor necrosis factor) – α signaling pathway which is mainly involved in regulation of immune cells. Fujiki and Suganuma (2011) revealed that chemical tumor promoters such as MCs are inducers of TNF-α in the cells of target tissues and that TNF-α is an endogenous tumor promoter (Fujiki and Suganuma, 2011).

From the pathway search, it is evident that MCLR/MCRR exposure has affected intestine tissues at the molecular level, which has manifested itself at the functional level as shown from results obtained for metabolite analyses.

8.3.3 Gills

Gill tissues showed the highest amount of significantly up/down regulated gene transcripts in the case of both MCLR and MCRR exposure among the exposed organs as compared to controls. This observation is understandable since in balneation route of exposure, the first biological contact point of toxins taken up by the fish would be gills (oxygen exchange happens at the gill interface). As a consequence, it is expected that the toxins, MCLR/MCRR, would have the longest residence time in gill tissues. This is unlike, other exposure routes, wherein, the toxins would go through the digestive system, reach liver (major detoxification site for MCs) and then migrate to other organs through hepatic circulation (Figure 8.1). Although liver is the detoxification organ, higher concentrations (or higher accumulation of MCs in fat tissues) would cause the degeneration of the hepatocytes and tumor promotions since MCs are, by nature, hepatotoxins (Carmichael, 1997). This is the reason why most of the literature reported on MCs is based on toxicological investigations done on liver tissues in various animal models. Exposure of zebrafish as modeled organisms to extracellular MCs via balneation route has not been extensively studied in the past. Therefore, toxicological information is restricted only to exploratory experiments on biochemical defense systems i.e. antioxidant enzyme levels after the exposure in fish organ systems. It is interesting to note that following extracellular MCs exposure to zebrafish, gills seem to be the most

highly affected organ instead of liver tissues as reported by other research groups in other animal models.

As mentioned earlier, 2354 genes were significantly affected in gill tissues, out of which 605 genes transcripts were common between MCLR and MCRR exposed zebrafish gills (See supplementary section). 1217 gene transcripts were only affected in the case of MCRR exposure while 532 gene transcripts were exclusively seen in the case of gill tissues exposed to MCLR (Figure 8.12, 8.13, 8.14) ($p < 0.05$ (corrected), fold change >2).

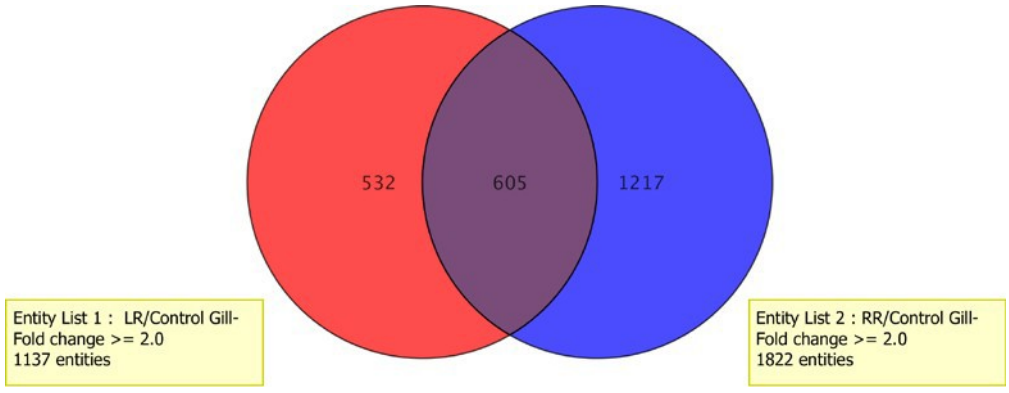

Figure 8.12: Venn diagram showing the distribution of significant gene transcripts in gill tissues exposed to MCLR/MCRR ($p < 0.05$ (corrected), fold change >2)

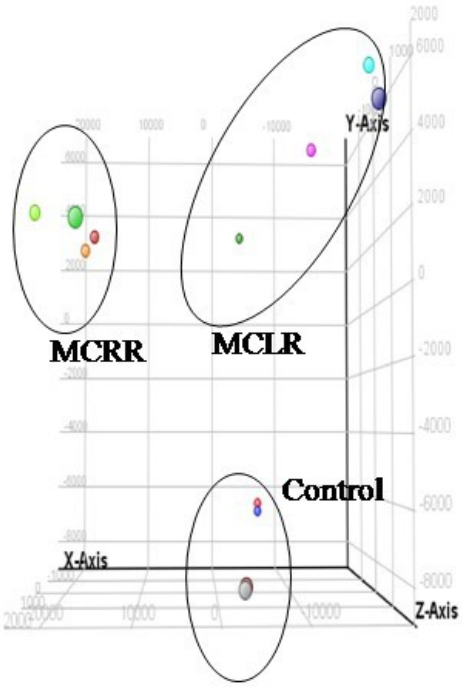

Figure 8.13: PCA plot showing the clustering of the three groups namely control, MCLR treated and MCRR treated gill tissue gene transcripts

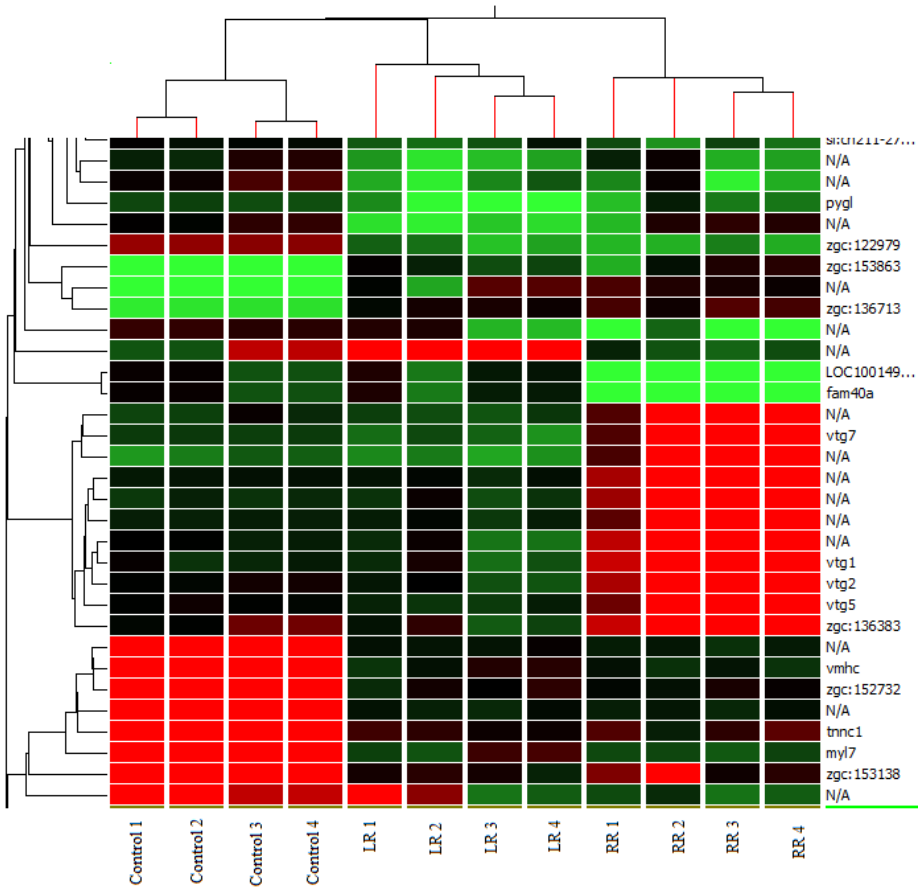

Figure 8.14: Heat map showing the difference in gene expression in a selected cohort of gill gene transcripts between MCRR and control group (■ Minimum intensity of gene probe; ■ Medium intensity of the gene probe; ■ Maximum intensity of gene probe)

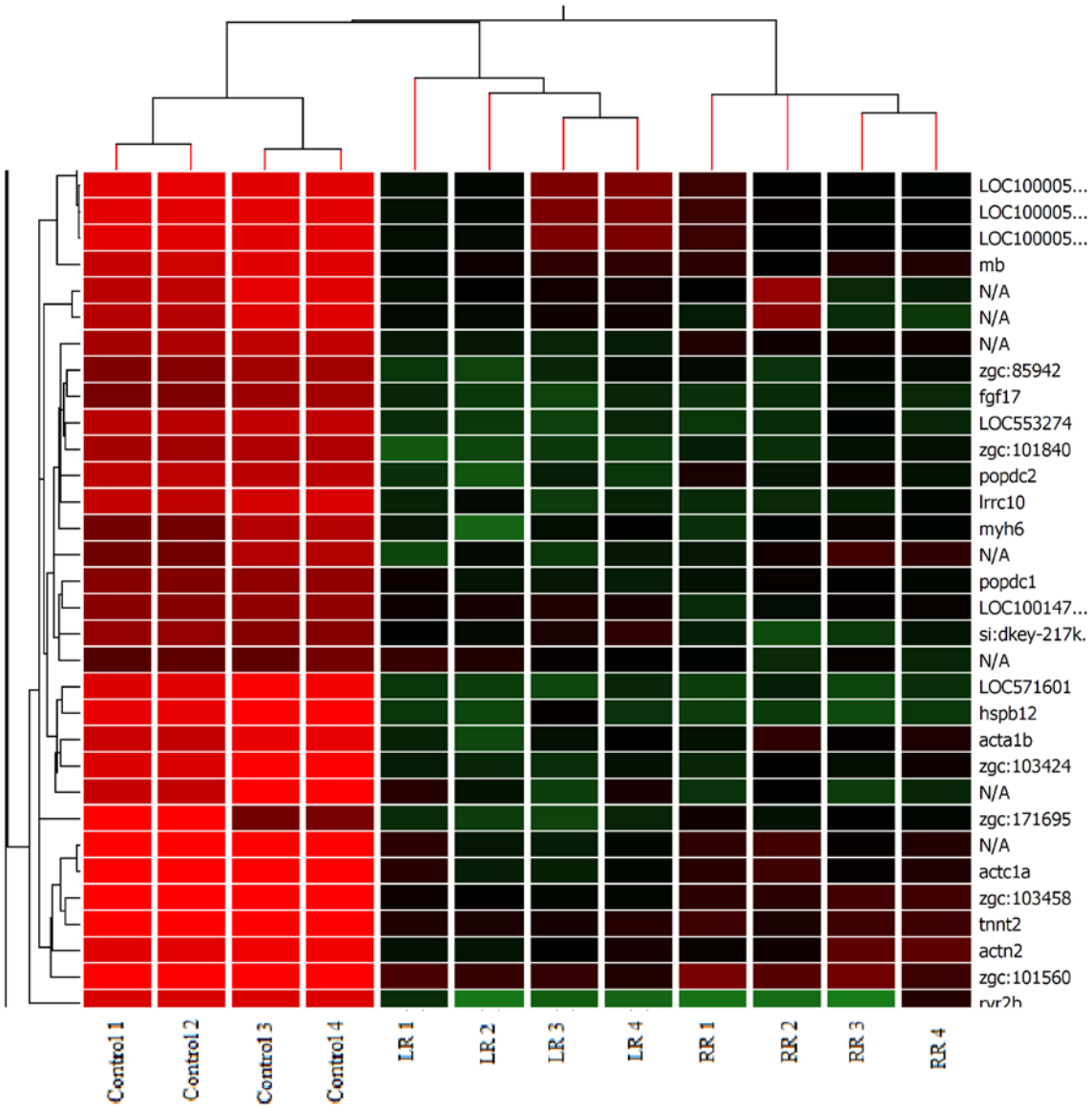

Figure 8.15: Heat map showing the difference in gene expression in a selected cohort of gill gene transcripts between MCRR and control group (Minimum intensity of gene probe; Medium intensity of the gene probe; Maximum intensity of gene probe)

The biochemical pathway search resulted in a long list of pathways which seemed to be affected due to the up/down regulation of gene transcripts in gill tissues. As many as 11 affected gene entities matched certain biochemical pathways (Table 8.3). Out of the

matched gene entities in biochemical pathways, a number of them belong to cell signaling pathways, some of which are common with the ones observed in brain tissues and intestine (Figure 8.16 and 8.17). Interestingly, carbohydrate and lipid metabolisms were also affected by the gene transcripts (Figure 8.18- 8.21). This finding is in line with the results obtained from metabolite analyses as discussed in chapter 7. These results suggest that deregulated gene transcripts did affect the tissues at the functional level, thus disrupting the entire metabolism. Apart from that, hormone related pathways were also affected, which was also seen in the case of metabolite analyses. However, some of the pathways were affected only by the gene transcripts originating from the MCLR exposure; not by MCRR and vice-versa. This is an interesting observation since both the toxins are very similar in their chemical structures, with the difference in only one amino acid. Moreover, this trend was not observed in other tissues in the present set of experiments. This difference between MCLR and MCRR in terms of their effects on gene expression could be due to their difference in toxicity potentials as discussed in previous sections (Figure 8.14 and 8.15).

Table 8.3 Affected biochemical pathways in gills tissue upon exposure to MCLR/MCRR

Pathway	Matching gene identities	Toxins involved
Dr_canonical_wnt_-_zebrafish_WP566_40794	11	LR/RR
Dr_Myometrial_Relaxation_and_Contraction_Pathways_WP1321_47925	11	LR/RR
Dr_ERK1_-_ERK2_MAPK_cascade_WP402_40778	10	LR/RR
Dr_Calcium_Regulation_in_the_Cardiac_Cell_WP1365_47926	10	LR/RR
Dr_MAPK_signaling_pathway_WP1337_47927	9	LR/RR
Dr_BMP_signaling_pathway_WP211_40771	9	LR/RR
Dr_G1_to_S_cell_cycle_control_WP445_40800	8	RR
Dr_FGF_signaling_pathway_WP152_40834	8	LR/RR
Dr_noncanonical_wnt_pathway_WP215_40786	8	LR/RR
Dr_TGF-beta_Receptor_Signaling_Pathway_WP1367_40788	8	LR/RR
Dr_IL-3_Signaling_Pathway_WP1359_40782	8	RR
Dr_DNA_Replication_WP451_40847	7	RR
Dr_Insulin_Signaling_WP1313_47923	7	RR
Dr_Striated_Muscle_Contraction_WP1316_40862	7	LR/RR
Dr_TNF-alpha_NF-kB_Signaling_Pathway_WP1369_40798	7	LR/RR
Dr_Cell_cycle_WP1393_40775	7	RR
Dr_neural_crest_development_WP548_40860	6	LR/RR
Dr_Endochondral_Ossification_WP1383_40811	6	LR/RR
Dr_IL-6_Signaling_Pathway_WP1322_40783	6	LR/RR
Dr_EGFR1_Signaling_Pathway_WP1323_40845	5	RR
Dr_Id_Signaling_Pathway_WP1374_40861	5	RR
Dr_Proteasome_Degradation_WP267_40797	5	RR
Dr_Nodal_signaling_pathway_WP341_29185	5	RR
Dr_Adipogenesis_WP1331_40773	5	LR/RR
Dr_Toll-like_receptor_signaling_pathway_WP1384_40839	5	LR/RR
Dr_Nodal_signaling_pathway_WP341_40857	5	RR
Dr_FAS_pathway_and_Stress_induction_of_HSP_regulation_WP511_47922	4	LR/RR

Pathway	Matching gene identities	Toxin involved
Dr_Integrin-mediated_cell_adhesion_WP1386_40815	4	RR
Dr_B_Cell_Receptor_Signaling_Pathway_WP1354_40807	4	LR/RR
Dr_Glycolysis_and_Gluconeogenesis_WP1356_40817	4	LR/RR
Dr_T_Cell_Receptor_Signaling_Pathway_WP1345_40851	4	RR
Dr_Wnt_Signaling_Pathway_WP1325_40870	4	RR
Dr_Glycogen_Metabolism_WP1388_40801	3	LR/RR
Dr_mRNA_processing_WP467_40828	3	RR
Dr_Cytoplasmic_Ribosomal_Proteins_WP324_40804	3	RR
Dr_Androgen_Receptor_Signaling_Pathway_WP1348_47924	3	LR/RR
Dr_p38_MAPK_Signaling_Pathway_WP1363_38231	3	LR/RR
Dr_p38_MAPK_Signaling_Pathway_WP1363_40871	3	RR
Dr_TGF_Beta_Signaling_Pathway_WP1370_40848	3	RR
Dr_Ovarian_Infertility_Genes_WP1340_40820	3	RR
Dr_Keap1-Nrf2_WP1332_46422	3	RR
Dr_Senescence_and_Autophagy_WP1378_47914	3	LR/RR
Dr_Hypertrophy_Model_WP1327_40824	3	LR/RR
Dr_Apoptosis_WP1351_47917	3	LR/RR
Dr_Oxidative_Stress_WP1372_40812	3	RR
Dr_Regulation_of_Actin_Cytoskeleton_WP1380_47916	3	RR
Dr_IL-2_Signaling_Pathway_WP1319_40829	2	RR
Dr_Translation_Factors_WP248_40813	2	RR
Dr_G13_Signaling_Pathway_WP1381_40816	2	RR
Dr_G_Protein_Signaling_Pathways_WP1371_40823	2	LR/RR
Dr_Biogenic_Amine_Synthesis_WP154_40837	2	RR
Dr_Wnt_Signaling_Pathway_WP1349_40790	2	LR/RR
Dr_Delta-Notch_Signaling_Pathway_WP1382_40791	2	LR/RR
Dr_Circadian_Exercise_WP562_40779	2	RR
Dr_Signaling_of_Hepatocyte_Growth_Factor_Receptor_WP444_40850	2	RR
Dr_TCA_Cycle_WP19_40831	2	RR
Dr_Electron_Transport_Chain_WP1339_35269	2	RR

Pathway	Matching gene identities	Toxin involved
Dr_One_Carbon_Metabolism_WP1355_40846	2	RR
Dr_Wnt_Signaling_Pathway_and_Pluripotency_WP1344_47920	2	LR/RR
Dr_IL-4_signaling_Pathway_WP1376_40873	2	RR
Dr_Diurnally_regulated_genes_with_circadian_orthologs_WP1379_40855	2	RR
Dr_Alpha6-Beta4_Integrin_Signaling_Pathway_WP1329_40859	2	LR/RR
Dr_SIDS_Susceptibility_Pathways_WP1377_40769	2	RR
Dr_cytochrome_P450_WP1390_48312	2	LR/RR
Dr_methylation_WP1334_40852	2	RR
Dr_Triacylglyceride_Synthesis_WP1347_48309	2	RR
Dr_Hypothetical_Network_for_Drug_Addiction_WP1333_40814	3	LR
Dr_Type_II_interferon_signaling_(IFNG)_WP1350_40774	2	LR
Dr_ErbB_signaling_pathway_WP1366_40818	2	LR
Dr_Eicosanoid_Synthesis_WP1318_40827	1	LR
Dr_Selenium_metabolism_Selenoproteins_WP1358_40805	1	LR
Dr_Nucleotide_GPCRs_WP1364_40865	1	LR
Dr_Cholesterol_Biosynthesis_WP1387_40849 0.47059685	1	LR/RR

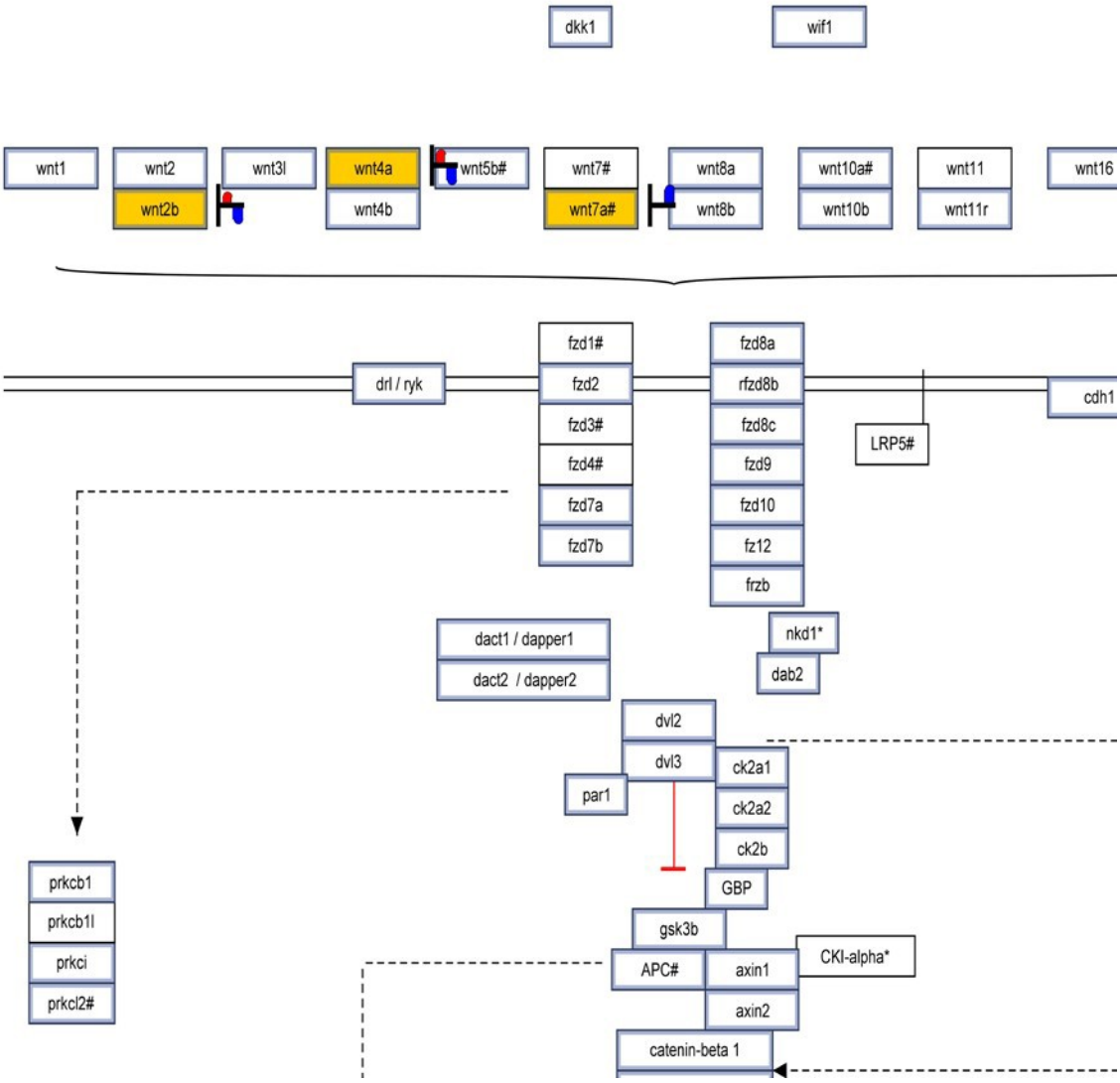

Figure 8.16 (a): Dr_canonical_wnt_-_zebrafish_WP566_40794 in zebrafish gill tissues exposed to MCLR. Gene transcripts (marked in yellow) are significantly up/down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCLR exposed gene transcripts)

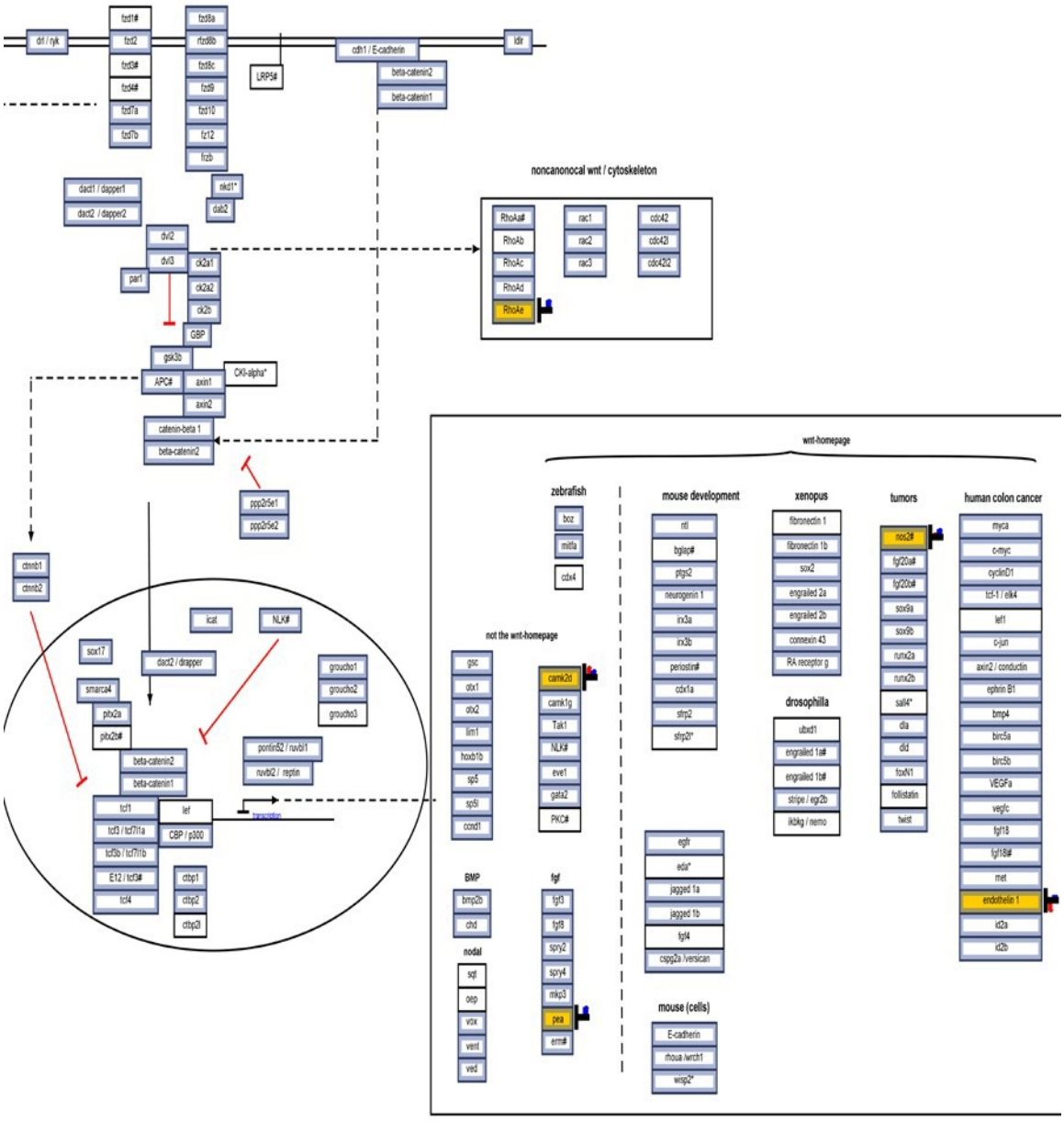

Figure 8.16 (b): Dr_canonical_wnt_-_zebrafish_WP566_40794 in zebrafish gill tissues exposed to MCLR. Gene transcripts (marked in yellow) are significantly up/down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCLR exposed gene transcripts)

As mentioned earlier, cell signaling pathways are crucial to cell death, cell proliferation, cell differentiation and a number of other key cellular processes. Maintaining control of

cellular functions is a critical undertaking in the development of any multicellular organism. Following an exposure with environmental pollutants, healthy cells in the exposed organism may be subjected to a series of new challenges that affect the maintenance of homeostasis. The cell must sense these homeostatic cues and make appropriate responses to ensure that vital functions such as proliferation, apoptosis, or differentiation, are properly orchestrated. Alternatively, or concomitantly, cells may mount an adaptive response where enzymes involved in catabolism are induced, or cellular defenses are activated (Ramos and Weber, 2010). The induction of cytochrome P-450 enzymes is a prime example of a cellular adaptive response to toxic injury. These adaptive responses lead to the processes which are termed as cellular signaling and the pathways that link the sensors (i.e., receptors) to the effectors (i.e., transcription factors, enzymes) of cell fate, the signal transduction cascade (Alder et al., 1999). In the present case, as can be seen from Table 8.3, Figure 8.16 and 8.17, the entire signal transduction cascade has been activated including the induction of cytochrome-P450 enzymes. Induction of this pathway was also seen in the case of metabolite analyses (Chapter 7). In general, alteration of signal transduction pathways may lead to various consequences.

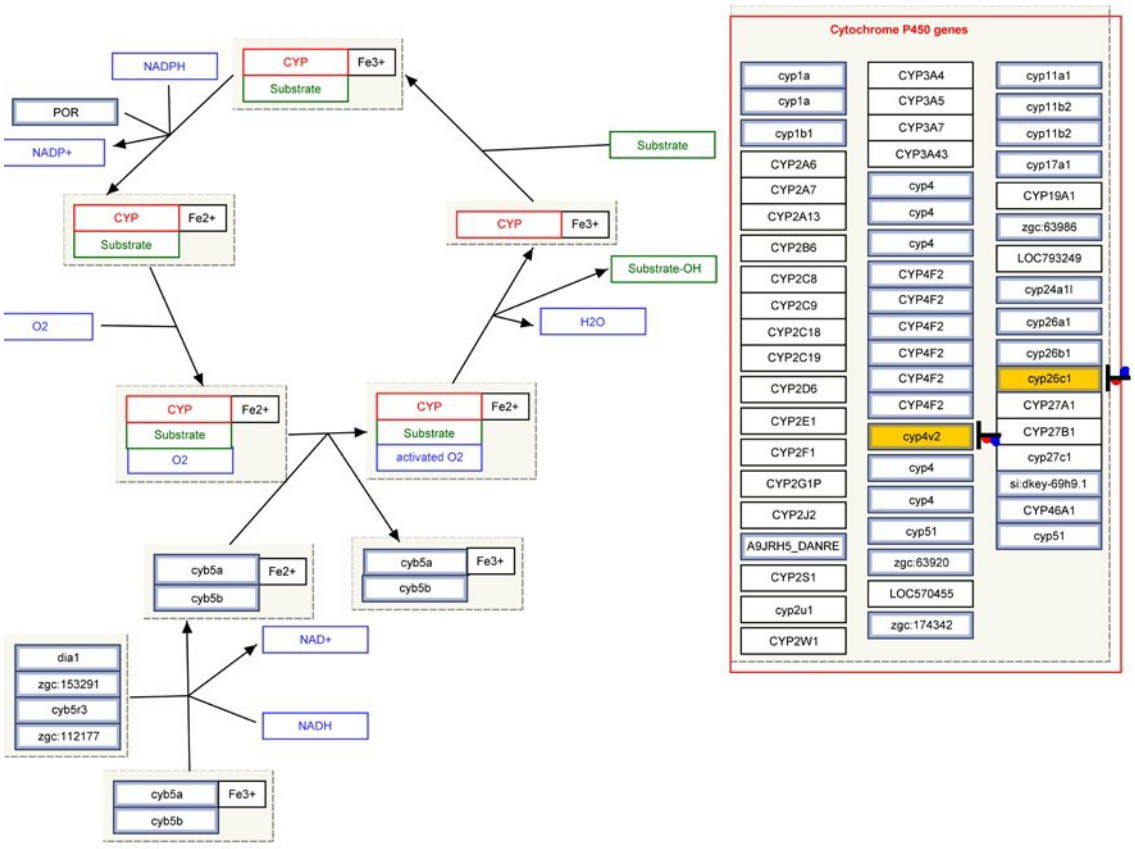

Figure 8.17: Dr_cytochrome_P450_WP1390_48312 in zebrafish gill tissues exposed to MCRR. Gene transcripts (marked in yellow) are significantly up/down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCRR exposed gene transcripts)

Firstly, many proteins involved in the signal transduction cascade are highly conserved across diverse species. Conservation of a protein sequence generally implies that a given protein plays an important biological role. It is likely that alteration in this protein's function via an environmental pollutant, MCs in this case, may have dire consequences. Secondly, dysregulation of several signal transduction pathways has been associated with tumorigenesis (Hunter 1997). As mentioned before in earlier sections, there are signaling pathways which are associated with tumor promoters that were observed in the present

study. Tumor promotion has been associated with acute exposure of MCs as confirmed by histological examinations. However, from the present study, it is evident that slow tumor promotion through the disturbance in the signaling network could be one of the toxicological implications of exposure to extracellular MCs as well.

Apart from cell signaling pathways, oxidative stress and related pathways (selenium metabolism) are also affected by the exposure in gill tissues (Table 8.3). Oxidative stress is an important by-product of the toxic response and can affect many signaling processes and ultimately the cell fate. There is a possibility that oxidative stress could also be responsible for activation of signal transduction cascade leading to tumor promotion.

Carbohydrate (mainly glucose) and lipid metabolisms were also affected by the exposure of zebrafish to MCLR/MCRR (Table 8.3, Figure 8.18-8.21). Most of the affected biochemical pathways were also detected by the metabolite analyses. This shows that the effects at transcriptional levels were translated at the functional level as well. Most of these pathways are directly, or indirectly dependent on protein phosphatases which are responsible for activation/ deactivation of these pathways, thus, controlling and balancing the flux. Since MCs are known to be inhibitors of these protein phosphatases, disruption in the metabolism of these macromolecules is expected in the case of MCs exposure. However,

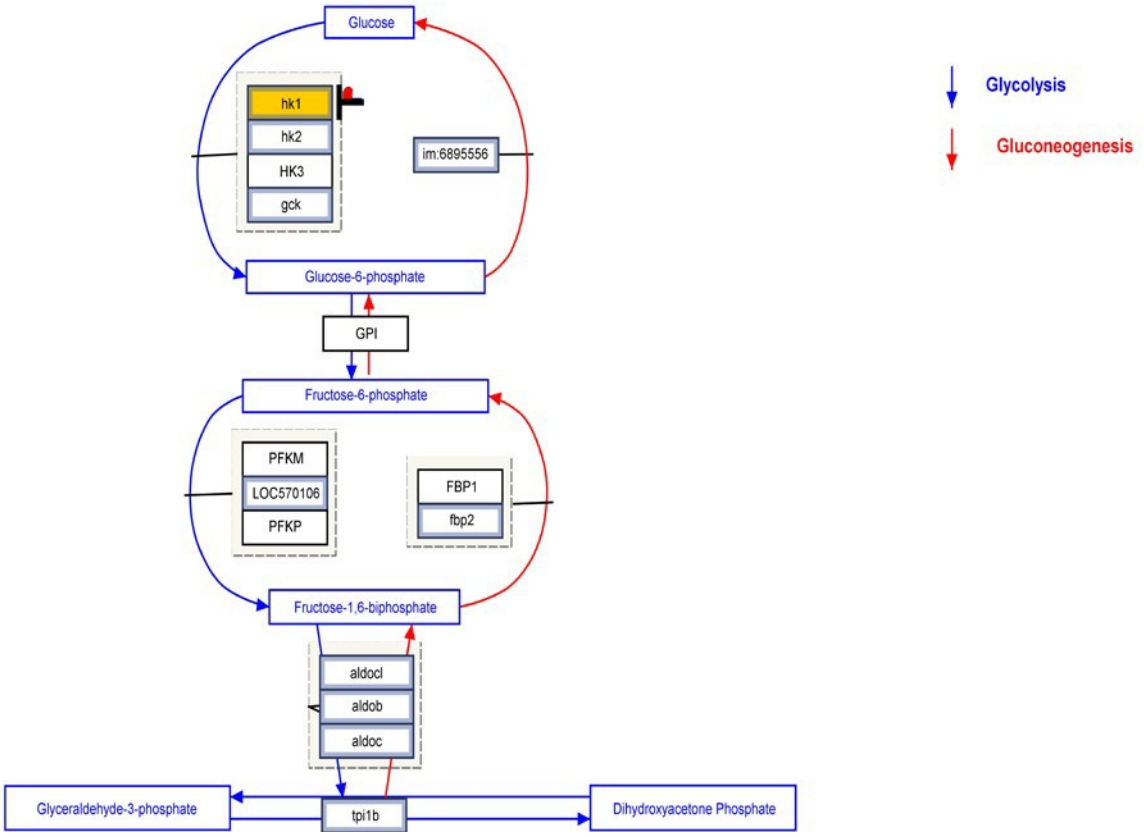

Figure 8.18 (a): Dr_Glycolysis_and_Gluconeogenesis_WP1356_40817 in zebrafish gill tissues exposed to MCLR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCLR exposed gene transcripts)

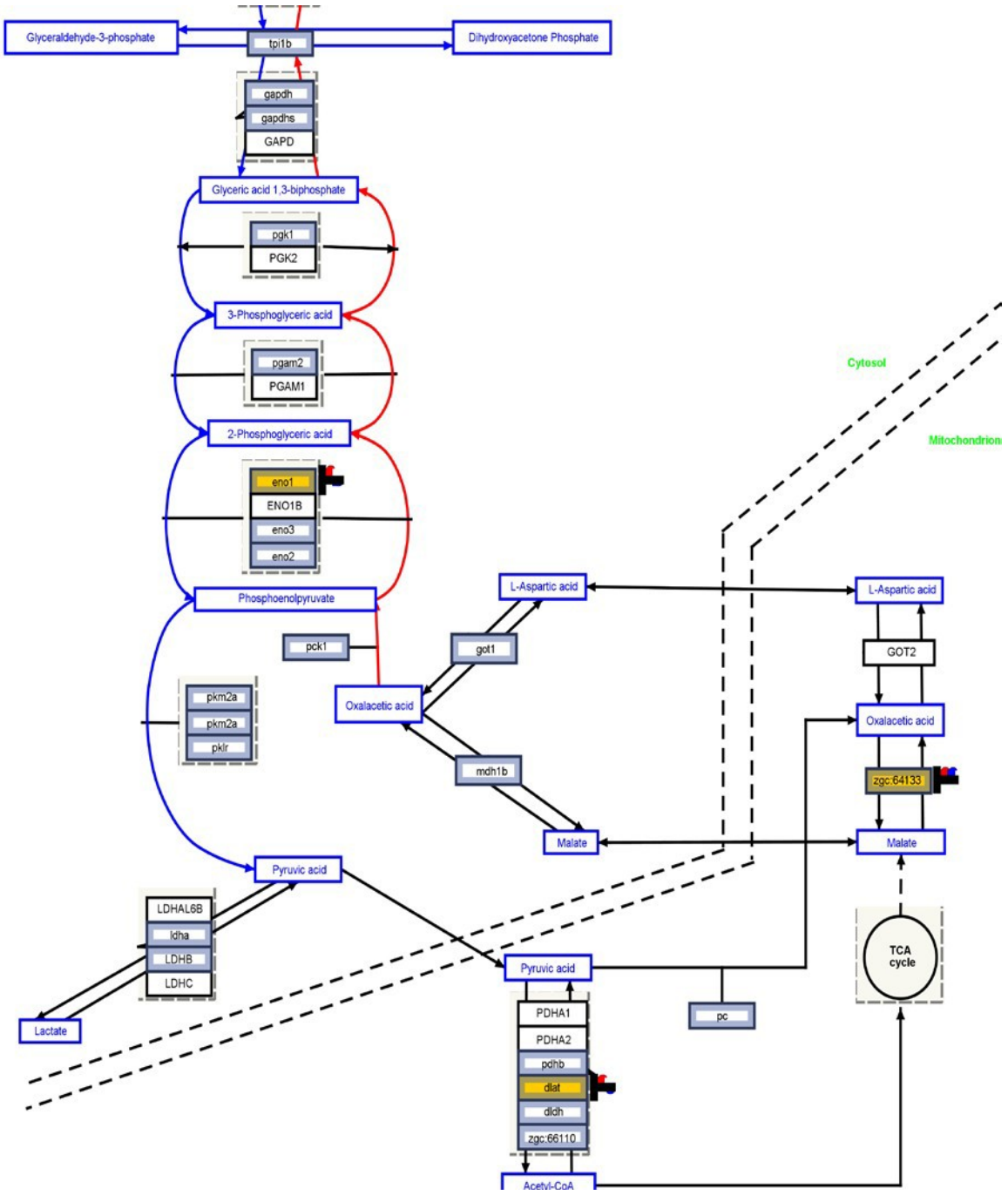

Figure 8.18 (b): Dr_Glycolysis_and_Gluconeogenesis_WP1356_40817 in zebrafish gill tissues exposed to MCLR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCLR exposed gene transcripts)

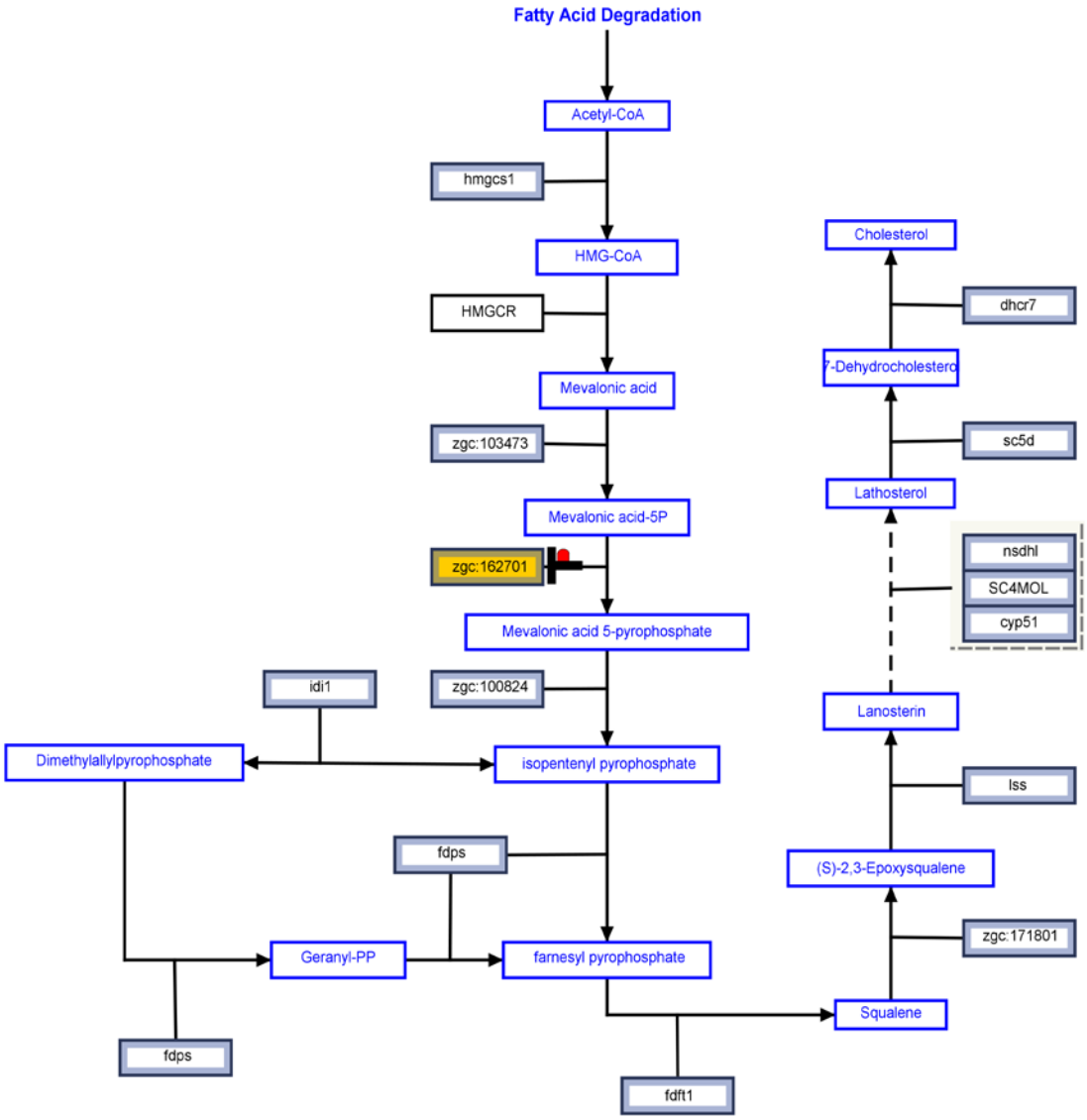

Figure 8.19: Dr_Cholesterol_Biosynthesis_WP1387_40849 in zebrafish gill tissues exposed to MCRR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCRR exposed gene transcripts)

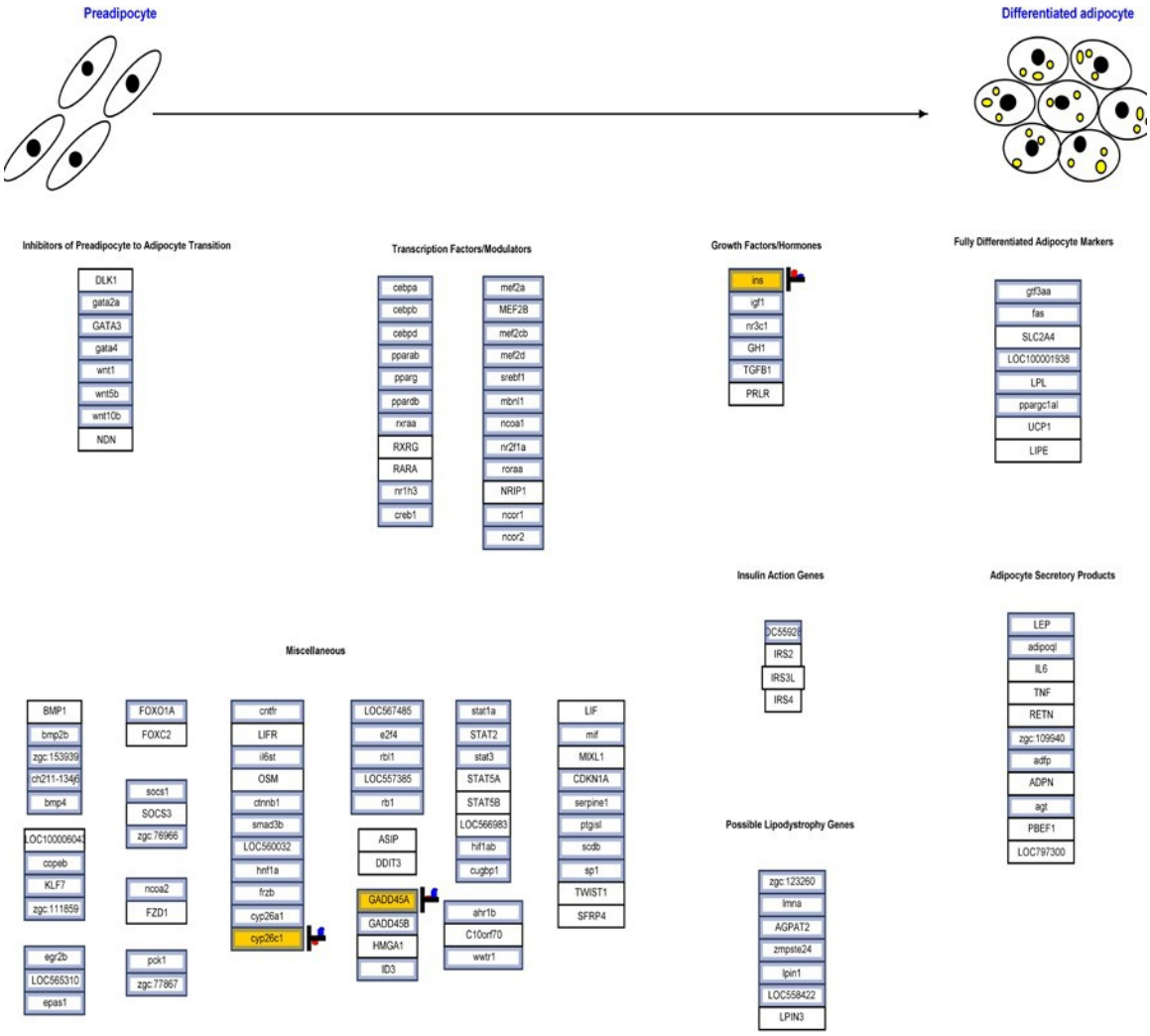

Figure 8.20: Dr_Adipogenesis_WP1331_40773 in zebrafish gill tissues exposed to MCRR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCRR exposed gene transcripts)

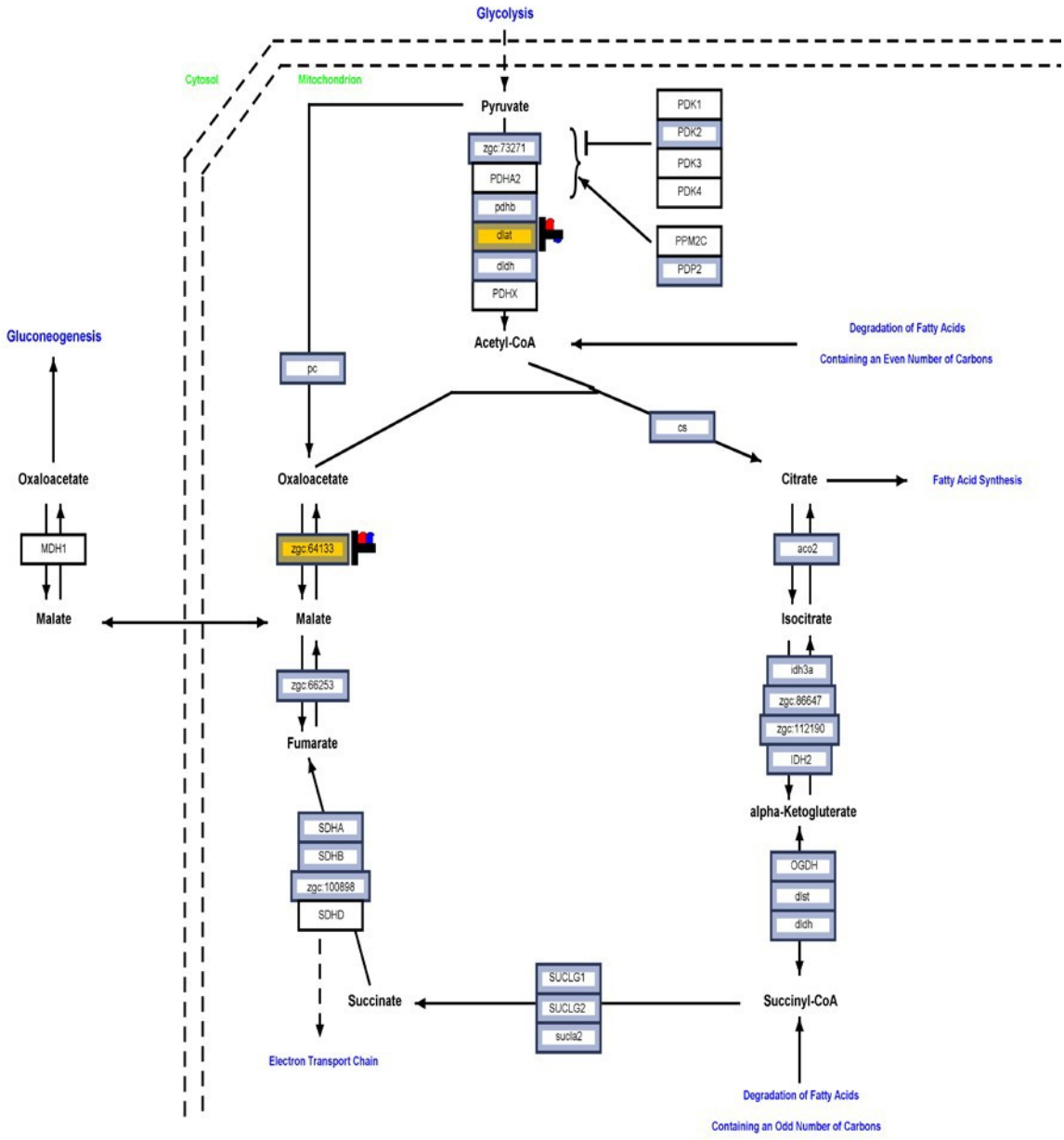

Figure 8.21: Dr_TCA_Cycle_WP19_40831 in zebrafish gill tissues exposed to MCRR. Gene transcripts (marked in yellow) are significantly down regulated as compared to control zebrafish intestine tissues (indicated in the bar next to the gene transcript; ■ intensity of control gene transcripts, ■ intensity of MCRR exposed gene transcripts)

as discussed earlier, cellular machinery can adapt itself to the toxic injury in certain cases and correct the metabolic changes. In the present study, it is evident that cells did not show an adaptive response even though the concentration of MCLR/MCRR in the water phase was much lower than the reported LD50 for aquatic organisms. Chronic and continued exposure to MCLR/MCRR could explain this observed phenomenon, wherein no lethality was observed, but there is a slow and sustained disruption of the cellular and metabolic machinery.

From the metabolite analyses and results from the present experiments, the endocrine disrupting role of MCLR/MCRR has been demonstrated. Endocrine disruption could have either estrogenic mode, or androgenic modes of action. In gill tissues, androgen-disruptive role of MCLR/MCRR could also be observed. Endocrine-disruptive role in terms of estrogenic modes of action has been described well by the study done on zebrafish larva (Rogers et al., 2011). However, the present study suggests the possible androgen disruption that could occur due to the chronic exposure to MCLR/MCRR.

In contrast to estrogenic modes of action, relatively little is known about how androgenic/antiandrogenic endocrine disrupting chemicals at environmentally relevant concentrations affect male reproductive tract health. Androgens mediate a wide range of developmental and physiological responses in the male and are crucial for testicular and accessory sex gland development and function, pubertal sexual maturation in multiple organs, maintenance of spermatogenesis and maturation of sperm, male gonadotropin regulation through feedback loops and various male secondary characteristics such as bone mass, musculature, fat distribution (Zirkin, 2010). Disruption in the pathways

connected to androgens could lead to dire consequences in terms of reproductive behavior of affected organisms. This disruption could have massive ecological impacts as discussed in earlier chapter (Chapter 7).

8.4 Conclusion

From the present data, it can be concluded that chronic exposure of zebrafish to extracellular MCs leads to severe toxicological implications affecting the tissues at the transcriptional level. Although changes occurring at the transcriptional level may not always translate completely to show an apparent change at the metabolic level, in the present study, most of the biochemical pathways that were affected at the metabolite level were rooted at the molecular level. Results from the present set of experiments are consistent with those discussed in previous chapters. Integration of the results from both 'omics' approaches (i.e. transcriptomics and metabolomics) could help in effective identification of potential biomarkers which could be used in evaluating the toxicological impact of extracellular MCLR and MCRR exposure on zebrafish in a holistic way.

CHAPTER 9

SUMMARY AND CONCLUSIONS

9.1 Summary

Frequent occurrences of harmful algal blooms (HABs) in surface waters and reservoirs pose a challenge in the production of safe drinking water. HABs are of major concern worldwide because of the production of a vast array of secondary metabolites with diverse bioactivities, known as cyanobacterial toxins or cyanotoxins. These cyanotoxins include potent hepatotoxins, neurotoxins, dermatotoxins, and cytotoxins which pose serious health risks to aquatic organisms as well as to human beings. Among the variety of toxins, hepatotoxic microcystins (MCs) are the group of toxins that were widely studied by researchers because of its high solubility, stability and persistence in natural resources. About 80 variants of these MCs are known to the scientific community. Out of them, MCLR and MCRR are the most frequently reported worldwide. MCLR and MCRR are released into the water bodies upon lysis of algal blooms. Both of them are released from the same strains of cyanobacteria. MCLR is the most toxic among all the variants known to date. It is most lethal to aquatic and human ecosystems. WHO has set a drinking water guideline in terms of MCLR equivalents as 1 μg L^{-1}. Due to the stability and chemical resistance of MCs, they persist in the water systems for fairly long periods of time. Although algal blooms are usually seasonal in nature, depending upon the favorable geographical conditions, MCLR and MCRR may persist in these aquatic systems. The presence of low doses of MCLR and MCRR could lead to chronic and sub-chronic exposures to the aquatic organisms. This exposure might not prove to be lethal to

aquatic species, but could still impair their metabolic systems leading to disturbance in ecosystem at a higher level of organization.

A number of studies have been conducted to-date on the health impacts of MCs on aquatic organisms. However, most of them have targeted the exposure to aquatic organisms with either intact cyanobacterial cells, or purified extracts through oral gavages and intraperitoneal injections. Very few studies documented the exposure of MCs to aquatic organisms through balneation mode (i.e. organisms bathed in the water containing MCs, which happens in case of lysis and continued sustenance of MCs as mentioned earlier). The studies on balneation exposure of aquatic species especially fish have reported the deleterious effects even though the concentrations are sub-lethal. However, there are no detailed, systematic investigations done on the health impacts of MCLR and MCRR present in low levels on any aquatic model organisms yet. In order to understand the possible hazards of MCLR/MCRR to the aquatic ecosystems and at large on human habitats, it is of prime importance to understand the modes and routes through which MCs could find their way through the organisms of concern. Apart from that, there is a need to understand the toxicity mechanisms and underlying health risks and implications which could result from exposure of MCLR/MCRR in a balenation mode. A deep insight into toxicity mechanisms could also result in potential biomarkers, which when monitored in sentential species could provide valuable information about the health of the aquatic ecosystems.

To fill the current knowledge gaps, this doctoral study was designed with a multi-factorial approach to conduct analytical and biological measurements at possible target

points at the level of organisms. For the present study, zebrafish was used as a model organism. Zebrafish has been considered to be a model for toxicological research dealing with environmental pollutants. Moreover, the entire genome of zebrafish has been sequenced which gives opportunities for in-depth exploration to provide valuable insights into understanding potential health impacts of environmental pollutants .

This study aimed at determination and toxicological evaluation of extracellular MCs present in reservoirs under tropical conditions using zebrafish as a model organism (Figure 9.1). MCLR and MCRR were used as the model MCs since they are widely studied and represent frequently occurring MCs in natural waters.

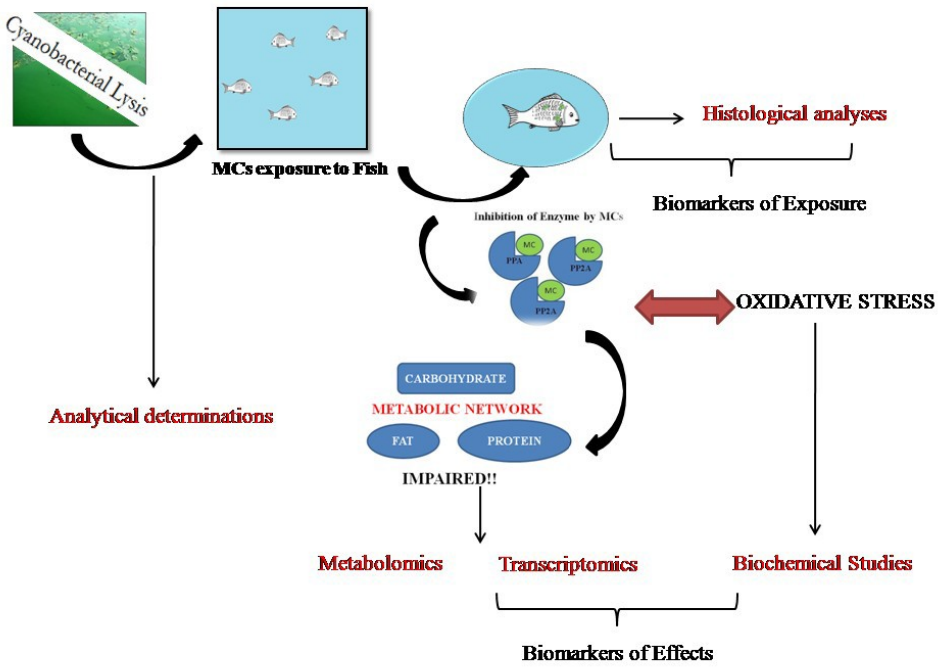

Figure 9.1: Schematic showing the workflow and rationale for the experiments conducted in this doctoral study.

The entire study was divided into two major components: analytical method development and biological/toxicological investigations. For the analytical component, a sensitive and selective method was developed for the analyses of MCLR and MCRR in the real water samples. Following the analytical method development, toxicological studies were done, aiming at identifying biochemical parameters, metabolic parameters and genomic parameters. These studies were conducted to understand the major toxicity mechanisms and the extent of deleterious health implications due to the exposure of aquatic organisms such as zebrafish to extracellular MCLR/MCRR. These studies also aimed at identifying possible specific biomarkers which could be used for further investigations by the scientific community.

9.2 Conclusions

The major conclusions drawn from this doctoral study are listed below**.**

9.2.1 Extraction and detection of MCs using ionic liquids:

A novel one step extraction/preconcentration method was developed for detection of MCLR and MCRR with low detection limits suitable for routine monitoring.

- Ionic liquid BMiM PF_6 was used for the determination of a MCLR and MCRR in natural waters. Extraction parameters such as sample pH, extraction temperature, extraction time, the amount of ionic liquid and the amount of extraction volume were investigated and optimized to achieve the maximum extraction efficiency.

- The calculated method detection limit was 0.03 µg L^{-1} (n=6) for MCLR and 0.09 µg L^{-1} for MCRR. The practical applicability of the developed technique was

demonstrated by analyzing water samples (n = 9) collected from three different sites in local reservoirs.

- Thus, the new analytical technique developed in this work is reliable, robust, and can be used for the routine monitoring of MCLR and MCRR in natural waters.

9.2.2 Biochemical changes in zebrafish organ systems following an exposure with MCLR/MCRR:

After developing and validating an analytical tool for detection of extracellular MCLR and MCRR, a comprehensive set of experiments were conducted for initial evaluation of biochemical marker reponse following a balneation exposure to MCLR/MCRR. Experiments were carried out to examine the dose-response of extracellular MCLR and MCRR toxicity in adult *Danio rerio* (zebra fish) under balneation conditions at various time points.

- The differential responses of SOD, GPx, GR, GST as biomarkers were assessed for oxygen mediated toxicity in liver, gills, intestine and brain tissues of zebra fish exposed to dissolved MCLR and MCRR (0.1 µg L^{-1} to 10.0 µg L^{-1}).

- To investigate the time related response of biomarkers, fish were sampled after 4, 7 and 15 days of exposure. Responses varied for (i) MCLR and MCRR (for certain groups), (ii) different enzymes at all time points, and (iii) different tissues. In general, most of the enzymes followed a bell shaped curve, with a spurt in activity at particular concentration.

- It was observed that upon exposure to MCLR and MCRR, some enzymes showed adaptive response after first time point wherein the enzyme activity increased in some tissues. The increase in enzyme activity suggested their cellular and metabolic adaptations to the continued stress and toxin exposure. Enzyme activities in general increased at lower concentrations ($\leq$5.0 µg L^{-1}) and decreased at higher concentrations ($\geq$5.0 µg L^{-1}). In general, initial stress conditions were observed in most of the tissue enzymes following the exposure to microcystins (MCs).

9.2.3 Qualitative biodistribution of MCLR/MCRR in zebrafish organs (Immuhistochemistry):

Although the exposure route was through gills, other organs were also affected upon exposure to MCLR and MCRR which was evident from the levels of antioxidant enzymes as described above. This supports the possibility of extracellular MCs entering the circulation and finding their way to the other organ systems. To understand it further, qualitative bio-distribution was studied using immunohistochemistry.

- Zebrafish were exposed to MCLR and MCRR (10.0 µg L^{-1}) under balneation conditions for 30 days. After the exposure, intact fish samples were studied using immunohistochemical procedures.

- Results from histochemical examinations revealed the presence of MCLR and MCRR in various tissues like intestine and liver apart from gill tissues. This showed that MCLR/MCRR after getting adsorbed at gill interface along with oxygen, reached the other tissues through systemic circulation.

Following the bio-distribution and preliminary biochemical experiments, in-depth analyses at metabolic and transcript (gene expression) levels were undertaken. This was done to understand the (i) possible in-depth toxicological implications of MCLR/MCRR exposure on the organism as a whole, (ii) to identify specific potential biomarkers.

9.2.4 Metabolomic evaluations of zebrafish organs following an exposure with MCLR/MCRR:

These experiments were undertaken to identify some novel pathways/ /metabolites which could serve as potential biomarkers as illustrated in Figure 9.2

- For the present set of experiments, zebrafish were exposed to MCLR and MCRR (10.0 µg L^{-1}) under balneation conditions for 30 days. After the exposure, fish organs were harvested and extracted for the metabolites.

- Results from metabolomic experiments showed that 33 biochemical pathways got affected in zebrafish organs following an exposure to MCLR/MCRR. Some of these biochemical and metabolic implications have been described and proposed in the literature and are known to the scientific community; however, some of the results obtained from these experiments were novel which have ecological implications. The major outcome from the 'omics' experiments are shown in Figure 9.3.

- Results showed that metabolites from lipid, carbohydrate and protein related biochemical pathways were perturbed in zebrafish organs especially in gills. Additionally, special class of compounds such as steroid hormones, glucocorticoids were also seen to be affected due to the exposure. Interestingly,

these hormones are involved in maintaining reproductive behavior of fish. Perturbation of the metabolites involved in their synthesis/degradation could, thus, lead to ecological implications.

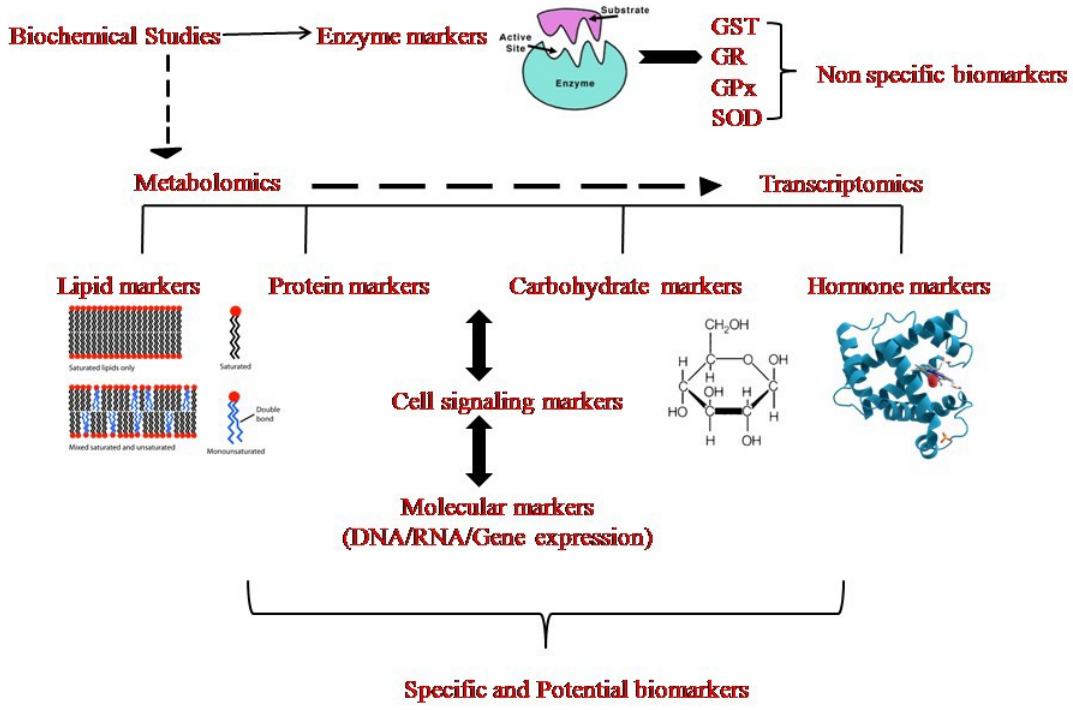

Figure 9.2: Schematic showing the workflow for hunt of potential biomarkers from different biological platforms used in this study

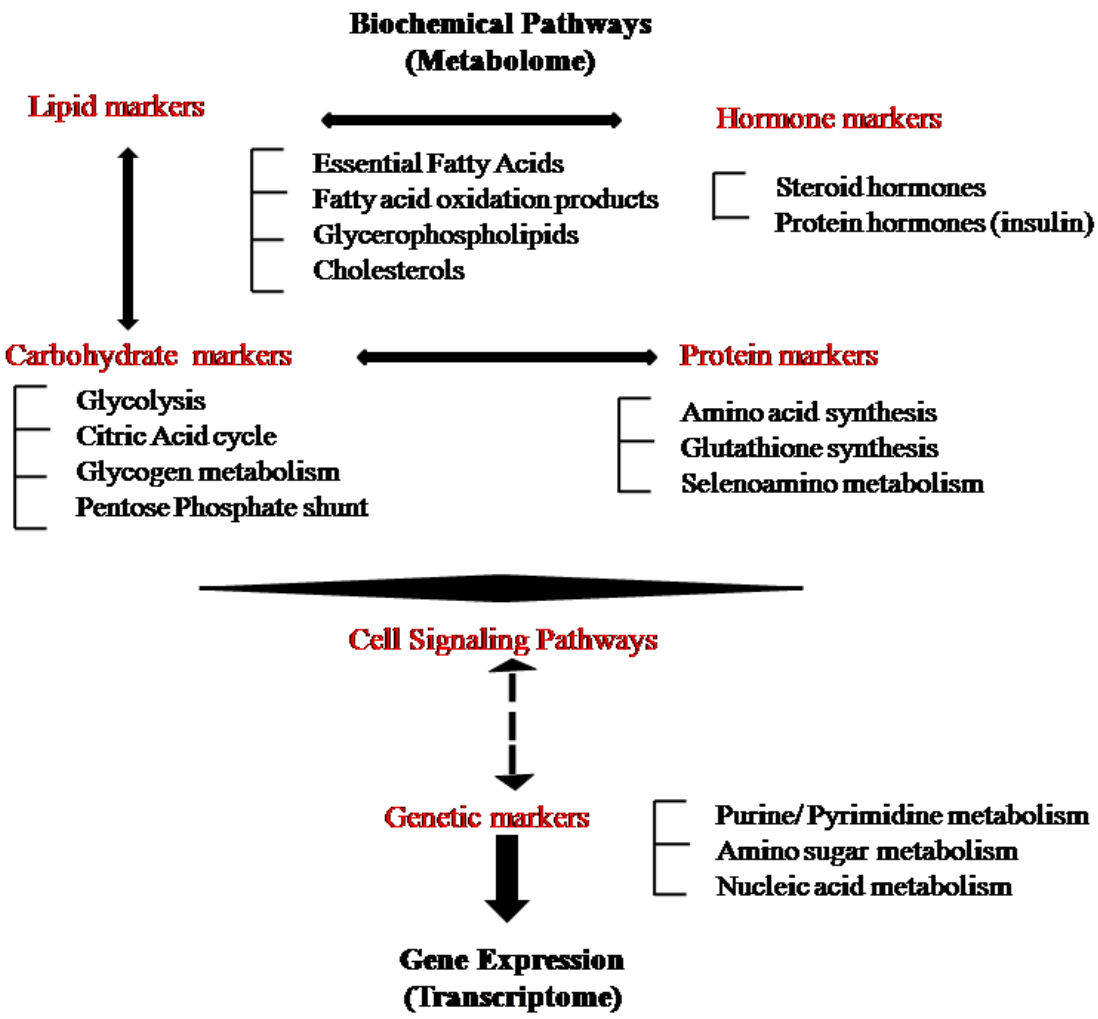

Figure 9.3: Schematic showing the potential pathways identified for biomarkers using omic approaches following an exposure of zebrafish with extracellular MCLR/MCRR

9.2.5 Transcriptomic evaluation of zebrafish organs following an exposure with MCLR/MCRR:

Metabolomic studies provided a valuable insight into several toxicity mechanisms and after effects of MCLR/MCRR exposure to zebrafish organs. Experiments also highlighted some potential biomarkers which could be further evaluated. Transcriptome or gene expression analyses were done to confirm and substantiate the results obtained from the metabolic profiles.

- For the present set of experiments, zebrafish were exposed to MCLR and MCRR (10.0 µg L^{-1}) under balneation conditions for 30 days. After the exposure, fish organs were harvested and were extracted for RNA.

- High-throughput analyses of 46,000 gene probes on the microarray were performed; gene transcripts that were significantly up/down regulated in zebrafish tissues were identified. A total of 2430 genes were significantly affected upon exposure to MCLR/MCRR ($p<0.05$, >2-fold change). Out of 2430 genes, 2354 genes were significantly affected in gill tissues with 23 being affected in brain and 53 being affected in intestine tissues.

- These affected gene transcripts belonged to the pathways involved in cell signaling networks (Figure 9.3). Most of the cells signaling pathways are very important for the maintenance of metabolism and other biochemical processes in the cells. Disruptions in cell signaling indicate metabolic perturbations at functional level. Results obtained from these experiments correlate well with the

metabolomic evaluations. Unique gene transcripts obtained from these experiments could result in biomarkers which could be used for monitoring purposes.

- A gene which is widely known as a biomarker of endrocrine disruption (vitellogenin, *vtg*) was also seen to be up regulated in response to the exposure to MCLR/MCRR in zebrafish tissues (brain, intestine, gills). This also directs at the endocrine disruptive abilities of MCLR/MCRR under chronic exposure.

The results obtained at the 'omics' and 'biochemical' levels provide a large knowlede-base of potential biomarkers , non specific and specific to the exposure of extracellular MCLR/MCRR to zebrafish. Since zebrafish is role model for toxicological research, most of these biomarkers could be extended to other organisms as well. Among these biomarkers, with further exploration, unique biomarker could be targeted for biomonitoring purposes using zebrafish, or any other sentential organism depending upon the locale of the study.

Classical texts define MCs, as acute hepatotoxins which often result in fatal outcomes when administered on test organisms. From the results presented in this doctoral study, it is evident that MCs are not mere 'hepatotoxins' as they were first introduced to the scientific community. MCs in sub-chronic exposure could also possess endocrine disruptive abilities. Apart from that, they could trigger signaling pathways at molecular level which initiate a cascade of events at metabolic level, thus hampering the entire system of the organism.

Hence, possible implications due to the chronic, low-dose exposure of MCLR/MCRR could lead to massive disturbance in the aquatic and human ecosystems.

Chapter 10

Recommendations for Further Research

Toxicological implications of extracellular MCs on aquatic organisms have not been studied extensively yet by the scientific community. Results from the present study clearly indicate the need for more comprehensive research in this area using different aquatic species to get deeper insights into the toxicological mechanisms that induce chronic health effects of MCs and to assess the risk associated with exposure to other extracellular MCs than those address in this thesis.

Apart from in-depth studies on toxicity of extracellular MCs, possible synergistic effects among other cyanotoxins should also be evaluated for their adverse health effect on aquatic organisms when present in extracellular phase. This type of toxicological investigations is important as , a number of other cyanotoxins have recently been reported to be released upon cyanobacterial lysis which could be equally or more toxic to the aquatic organisms than MCs. Moreover, the fate and transport of cyanotoxins in tropical reservoirs remain incompletely understood. Cynaotoxins may remain dissolved in the aqueous phase, or be attached suspended particulate matter; the partitioning of cyanotoxins between aqueous and particulate phases depends on many factors including the pH, ionic strength, and temperature of the aquatic systems. Fundamental laboratory investigations are needed to quantify this partitioning ratio for cyanotoxins which can then be included in water quality models for better characterization of their fate and transport. In addition, the mechanisms involved in the lyses of cyanobacteria and the subsequent release of cyanotoxins in aquatic systems remain uncertain. Controlled

laboratory experiments are needed in this research area too.

Moreover, presently there are not many research articles focused on the toxicity of MCs in conjunction with other environmental and biological factors (Figure 10.1). There is a strong need for extensive research in this area to understand the impact of MCs in a more holistic way. Other environmental factors of concern are climatic, physical, chemical or biological in nature which could affect the ultimate toxic potency of MCs to fish species. Without addressing the role of factors in MCs toxicity studies, the research results obtained cannot be subjected to extrapolation since they lack realistic significance. One of the most important factors to consider is the presence of several MC variants co-existing at a single point of time in an aquatic system. As discussed in this article, different MC variants have varied biological affinities i.e. their potential to enter a fish tissue would be different depending on the affinity of the ion transporters for that MC variant. When more than one MC variant is present in contaminated aquatic systems, there are possible chances of interference at the site of cell transporters-MC variant interaction. Uptake and toxicokinetics would then be driven by a different set of factors. The toxicity profiles thus obtained might not represent the toxicity profile when a single MC variant is administered. Apart from MC variants, a host of environmental pollutants find their way to the aquatic systems. Some of them are susceptible to degradation while others stay stable even in adverse conditions. All the major environmental pollutants exist along with the MCs variants upon the lyses of the bloom in the real waters. Interaction among the constituents of aquatic systems is plausible. Since an ecosystem is dynamic yet interlinked, these interactions could also affect the resultant toxicity of MCs on fish species. Hence, unless all the above-mentioned factors are taken into consideration, a

complete understanding the complex phenomenon of HAB and its impact on water quality occurring is not possible.

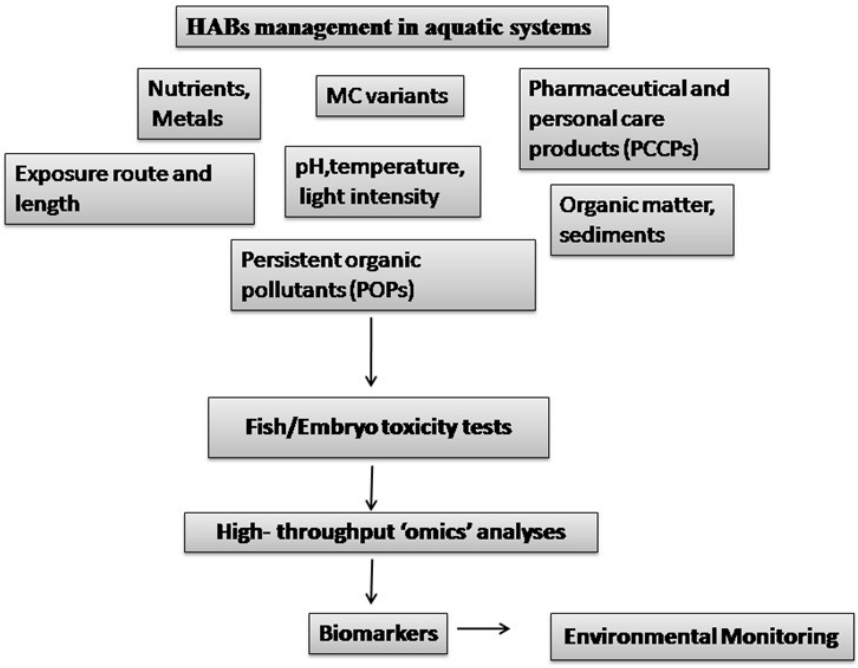

Figure 10.1: A schematic showing the work-flow for HABs management in aquatic systems

We have also explored the use of adsorption-based treatment methods for removal of MCLR and MCRR using environmental samples collected from local reservoirs (not included in the thesis, but the outcome of these studies is already published as can be seen from the list of publications included on page xiv). AOP-based (advanced oxidation processes) treatment methods are currently explored for complete mineralization of cynaotoxins with the use of photocatalysts and strong oxidants. However, it is not clear if these methods are effective in detoxifying water samples contaminated with cyanotoxins, which would depend on the nature and toxicity of by-products obtained. Appropriate cytotoxicity and genotoxicity assays should be developed for evaluating the toxicity of treated water samples.

Lastly, the identified pathways for potential biomarkers from this study should be used to build transgenic zebrafish lines for detecting the presence of MCs in the aquatic systems. Till date, transgenic lines for zebrafish exist for metals and certain other environmental pollutants, but not for cyanotoxins. Development of such zebrafish lines for MCs detection would help in risk management practices and thus could prevent massive ecological disturbance that may accompany a cyanobacterial bloom lysis.

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REPRESENTATIVE DATA INCLUDED IN APPENDICES

Metabolomic Studies

Table S 1: Significant metabolites ($p<0.05$, after correction; fold change > 2) from gill tissues of zebrafish detected and identified in all the experimental groups :

Non Polar – Postive mode

A) LR/C

M/Z	Common Name	Fold change	Chemical Formula	Pathway
384.262	Cholest-4-en-3-one; Cholestenone; 4-Cholesten-3-one	2.19	C27H44O	Steroid biosynthesis
286.210	Androstenedione; Androst-4-ene-3,17-dione; 4-Androstene-3,17-dione	2.02	C19H26O2	Steroid biosynthesis
399.367	L-Palmitoylcarnitine	2.53	C23H45NO4	Fatty acid metabolism

B) RR/C

M/Z	Compound name	Fold change	Chemical formula	Pathway
117.077	L-Valine; 2-Amino-3-methylbutyric acid	2.69	C5H11NO2	Valine,leucine, isoleucine degradation
780.614	3-Demethylubiquinone-9	2.80	C53H80O4	N/A
297.334	Tridemorph	2.27	C19H39NO	N/A

C) LR/RR

M/Z	Compound name	Fold Change	Chemical formula	Pathway
865.723	2-methylacetoacetyl-CoA	2.11	C26 H42 N7 O18 P3 S1	Valine,leucine, isoleucine degradation
386.569	cholesterol	2.20	C27 H46 O1	Steroid biosynthesis
458.459	5,10-methylene-THF	2.13	C20 H24 N7 O6	Glycerophospho lipid metabolism
943.792	feruloyl-CoA	2.0	C31 H44 N7 O19 P3 S1	Phenylalanine metabolism
929.743	caffeoyl-CoA	2.10	C30 H42 N7 O19 P3 S1	Phenylalanine metabolism
913.748	4-coumaroyl-CoA	2.22	C30 H42 N7 O18 P3 S1	Phenylalanine metabolism
853.664	3-hydroxybutyryl-CoA	2.12	C25 H42 N7 O18 P3 S1	Fatty acid oxidation
911.747	3-hydroxy-3-methyl-glutaryl-CoA	3.14	C27 H44 N7 O20 P3 S1	N/A
821.659	acrylyl-CoA	2.56	C24 H38 N7 O17 P3 S1	Valine,luecine, isoluecine degradation
932.760	indole-3-acetyl-CoA	2.12	C31 H51 N8 O17 P3 S1	N/A
791.640	UDP-3-O-(3-hydroxymyristoyl)glucosamine	2.24	C29 H51 N3 O18 P2	N/A
918.729	kaempferol 3-O-[6-(4-coumaroyl) -beta-D-glucosyl -(1->2)-glucosyl-(1->2)-beta-D-glucoside	2.34	C42 H46 O23	N/A
921.723	phenyllactatyl-CoA	3.45	C30 H50 N7 O18 P3 S1	N/A
887.734	salicyloyl-CoA	3.56	C28 H40 N7 O18 P3 S1	N/A
489.281	CDP-choline	2.34	C14 H27 N4 O11 P2	Glycerophospho lipid metabolism
930.809	(RS)-beta-phenylalanoyl-CoA	3.21	C30 H45 N8 O18 P3 S1	N/A
580.231	UDP-D-glucuronate	3.32	C15 H22 N2 O18 P2	Pentose and glucoranate interconversions
849.680	3-methylcrotonyl-CoA	2.34	C26 H42 N7 O17 P3 S1	Valine,leucine, isoluecine degradation
861.688	hexanoyl-CoA	2.45	C27 H42 N7 O17 P3 S1	Fatty acid oxidation; fatty acid elongation

Non Polar- Negative mode

A) LR/C

M/Z	Common name	Fold change	Chemical formula	Pathway
281.070	4-Hydroxyphenylacetylglutamic acid; Hydroxyphenylacetylglutamate	3.45	C13H15NO6	Tyrosine metabolism
354.159	Prostaglandin D1	4.32	C20H34O5	Arachidonic acid metabolism
352.223	Prostaglandin H2; 11alpha-Epidioxy-15-hydroxyprosta-5, 13-dienoate; PGH2	2.13	C20H32O5	Arachidonic acid metabolism
334.214	Prostaglandin A2; PGA2; Medullin	2.56	C20H30O4	Arachidonic acid metabolism
794.537	Ubiquinone-9; Ubiquinone-45; Coenzyme Q(9)		C54H82O4	Ubiquinone synthesis

B) RR/C

M/Z	Common name	Fold change	Chemical_Formula	Pathway
243.183	Cytidine	2.56	C9H13N3O5	Pyrimidine metabolism

C) LR/RR

M/Z	Common Name	Fold change	Chemical Formula	Pathway
696.551	2-Octaprenyl-3-methyl-6-methoxy-1,4-benzoquinone	2.78	C48H72O3	Ubiquinone synthesis
227.181	Deoxycytidine; 2'-Deoxycytidine	2.1	C9H13N3O4	Pyrimidine metabolism
766.626	di-trans,poly-cis-Undecaprenol; Undecaprenol	2.34	C55H90O	Peptidogylcan biosynthesis
320.235	(15S)-15-Hydroxy-5,8,11-cis-13-trans-eicosatetraenoate; 15(S)-HETE		C20H32O3	Arachidonic acid metabolism
312.231	(9Z,11E)-(13S)-13-Hydroperoxyoctadeca-9,11-dienoic acid; 11E-octadecadienoic acid	3.1	C18H32O4	Linoleic acid metabolism
171.116	2,3,4,5-Tetrahydrodipicolinate	3.2	C7H9NO4	Lysine biosynthesis
776.614	O-(4-Hydroxy-3,5-diidophenyl)-3,5-diiodo-L-tyrosine; L-Thyroxine	2.67	C15H11I4NO4	Tyrosine metabolism
243.183	Cytidine	2.45	C9H13N3O5	Pyrimidine metabolism
130.062	4-Methyl-2-oxopentanoate; 2-Oxoisocaproate	2.34	C6H10O3	Valine, luecine, isoluecine degradation

Polar – Positive mode

A) LR/C

M/Z	Common Name	Fold Change	Chemical Formula	Pathway
324.046	UMP; Uridylic acid; Uridine monophosphate;	5.67	C9H13N2O9P	Pyrimidine metabolism, peptidoglycan biosynthesis
480.477	1-Hexadecyl hexadecanoate	2.34	C32H64O2	N/A

B) RR/C

M/Z	Common_Name	Fold Change	Chemical Formula	Pathway
382.119	Farnesyl diphosphate	4.56	C15H28O7P2	Steroid biosynhesis
194.043	D-Glucuronate; Glucuronic acid	5.67	C6H10O7	Pentose and glucuronate interconversions, Starch and sucrose metabolism
194.043	L-Guluronic acid	3.11	C6H10O7	Pentose and glucuronate interconversions, Starch and sucrose metabolism

Polar- Negative

A) RR/C

Query M/Z	Common_Name	Fold Change	Chemical Formula	Pathway
382.119	Farnesyl diphosphate	5.43	C15H28O7P2	Steroid biosynthesis

B) LR/RR

Query M/Z	Common_Name	Fold Change	Chemical Formula	Pathway
448.324	Glutathionyl--aminopropylcadaverine	6.54	C18H36N6O5S	Glutathione metabolism

Table S2: Significant metabolites ($p<0.05$, after correction; fold change > 2) from intestine tissues of zebrafish detected and identified in all the experimental groups

Non Polar- Positive

A) LR/C

M/Z	Common Name	Fold Change	Chemical Formula	Pathway
236.094	Glycosminine	5.43	C15H12N2O	Drug metabolism-cytochrome P450
312.260	Eicosanoic acid; Arachidic acid	5.67	C20H40O2	Linoleic acid metabolism; Fatty acid biosynthesis